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The Effects Of Arsenic On Selenoprotein Biosynthesis

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THE EFFECTS OF ARSENIC ON SELENOPROTEIN BIOSYNTHESIS

by

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A thesis submitted in partial fulfillment of the requirements
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ABSTRACT

Arsenic contamination of drinking water is a real public health problem in certain areas of South-East Asia where chronic exposure has been correlated to higher rates of lung, skin, bladder, kidney, and liver cancer. Although arsenic carcinogenicity is well established, the mechanism by which it induces cancer is poorly understood. Recent evidence suggests that oxidative stress could be a possible mechanism for the carcinogenic effects of arsenic. Selenium, in the form of selenocysteine, is necessary for the activity of several enzymes with a role in the defense against reactive oxygen species (ROS), primarily thioredoxin reductases (TrxR) and glutathione peroxidases (Gpx). One of the key enzymes in the incorporation of Se into selenoproteins is selenophosphate synthetase (SPS). SPS catalyzes the activation of Se to selenophosphate, and is the first step in the pathway of selenoprotein biosynthesis. SPS contains a conserved vicinal dithiol motif (CXC) within a region of amino acids that have been predicted to be a selenium binding site. Our hypothesis is that arsenite inhibits new selenoprotein synthesis, thus indirectly increasing the level of ROS.

In this study we have developed a spectrophotometric assay for SPS. Using this assay, we have determined that arsenite inhibits SPS activity. Kinetic analysis of this inhibition showed that arsenite, a trivalent form of arsenic, acts as a competitive inhibitor with the substrate, sodium selenide. This inhibition of SPS could represent a potential molecular mechanism for oxidative stress induced upon arsenite treatment of human cell lines in culture. To further study the effects of trivalent arsenicals at a cellular level we decided to use a human keratinocyte cell line, HaCaT as a cell culture model. Our study showed that although arsenite does not alter cell proliferation

or protein synthesis, it specifically inhibits new selenoprotein synthesis. However, short term or long term exposure of HaCaT cells to arsenite failed to result in changes to Gpx and TrxR levels.

Since the radioisotope selenium used in labeling studies is selenite, these results indicate that an alternate source of selenium may bypass the inhibitory effects of arsenite. Future studies will focus on studying the effects of arsenicals on keratinocytes cultured in a defined medium allowing a better control of the selenium source.

This work is dedicated to my family. Their love and support permitted me to be where I am today.

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LIST OF ACRONYMS/ABBREVIATIONS

AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
As	Arsenic
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine Serum Albumin
Cys	Cysteine
DEAE	Diethylaminoethyl
Dio	Deiodinase
DMEM	Dulbecco's modification of Eagle's medium
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
DNPH	2,4-dinitrophenyl-hydrazine
DTNB	5,5'-dithio- <i>bis</i> -(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra-acetate
FAD	Flavin Adenine Dinucleotide
FBS	Fetal Bovine Serum
Gly	Glycine

Gpx	Glutathione peroxidase
GSH	Reduced Glutathione
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
IPTG	Isopropyl- β -D-Thiogalactopyranoside
MESG	2-amino-6-mercapto-7-methylpurine riboside
Met	Methionine
MetO	Oxidized Methionine
Msr	Methionine Sulfoxide Reductase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
NBT	Nitro Blue Tetrazolium
OD	Optical Density
PBS	Phosphate Buffered Saline
Pi	Inorganic Phosphate
PNP	Purine Nucleoside Phosphorylase
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Se	Selenium
SECIS	Selenocysteine Incorporation Sequence
SeCys	Selenocysteine
Sel	Selenoprotein

SPS	Selenophosphate Synthetase
TBS-T	Tris Buffered Saline-Tween
TLC	Thin Layer Chromatography
TrxR	Thioredoxin Reductase
UTR	Untranslated Region
XAS	X-ray Absorption Spectroscopy

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1- Importance of selenium

The metalloid selenium exists naturally in organic (e.g. selenomethionine, selenocysteine) and in inorganic forms (e.g. selenite, selenate, selenide) (Birringer M., 2002). Selenium compounds need to be metabolized into an inorganic precursor, selenide (NaHSe), before being inserted into proteins as the 21st amino acid selenocysteine that is essential for the catalytic function of selenoenzymes (Carlson B.A., 2004). Selenium is required in several mammalian enzymes with roles in defense against reactive oxygen species. These include thioredoxin reductase (TrxR), several isoforms of glutathione peroxidase (Gpx) and a selenium-dependent form of methionine sulfoxide reductase (SelR) (Tamura T., 1996, Arner, E. S., 2000, Lee S. R. et al., 2000, Flohe, L. et al., 1973, Brigelius-Flohe, R., 1999, Chambers, I. et al., 1986, Kryukov, G. V. et al., 2002). In a recent article, using a computational approach, the authors (Kryukov, G.V. et al., 2003) have shown that 25 genes encoding selenoproteins reside in the sequenced human genome (Table 1). All selenoproteins with known enzymatic functions contain selenocysteine in their catalytic center. Selenoproteins incorporate SeCys cotranslationally into the polypeptide chain by a complex mechanism (Low, S.C., 1996) that was only recently elucidated in mammalian system.

Table 1: Mammalian Selenoproteins

Selenoprotein	Common abbreviations	Key references
Glutathione peroxidase	GPx	
Cytosolic or classical GPx	cGPx, GPx-1	Mills G.C. 1957
Phospholipid hydroperoxide GPx	PHGPx, GPx-4	Ursini F. 1982
Plasma GPx	pGPx, GPx-3	Takahashi K. 1987
Gastrointestinal GPx	GI-GPx, GPx-GI, GPx-2	Chu F-F. 1993
Iodothyronine deiodinases		
50-deiodinase, type I	50DI	Behne 1990, Arthur 1990
50-deiodinase, type II	50DII	Davey J.C. 1995
5-deiodinase, type III	5-DIII	Croteau W. 1995
Thioredoxin reductases		
Thioredoxin reductase	TR	Tamura T. 1996
Mitochondrial thioredoxin reductase	TR-2	Lee 1999, Watabe 1999, Miranda-Vizuete 1999
Thioredoxin reductase homologs	SelZf1, SelZf2	Lescure A. 1999
Selenophosphate synthetase-2		Guimaraes M.J. 1996
15-kDa selenoprotein (T cells)		Gladyshev V.N. 1998
Selenoprotein P	SelP	Motsenbocker, M.A. 1984
Selenoprotein W	SelW	Vendeland S.C. 1995
Selenoprotein R	SelR	Kryukov G.V. 1999
Selenoprotein T	SelT	Kryukov G.V. 1999
Selenoprotein X	SelX	Lescure A. 1999
Selenoprotein N	SelN	Lescure A. 1999

1.2- Selenoprotein biosynthesis

The process by which selenocysteine is inserted via translation of a UGA codon (normally a stop codon) is well described in *Escherichia coli* (Bock, A., 1991, Leinfelder, W. et al., 1990), and is currently being characterized in mammals (Berry, M. J. et al., 1997, Berry, M. J. et al., 2002). A specific tRNA is first loaded with serine, and then transformed into selenocysteyl-tRNA using selenophosphate as the activated Se donor. The common UGA stop codon is recoded by SelB, a translation factor in the presence of a SeCys-charged tRNA and a 3' untranslated region (SECIS) (Figure 1) (Forchhammer, K., et al., 1990, Forster C. et al., 1990).

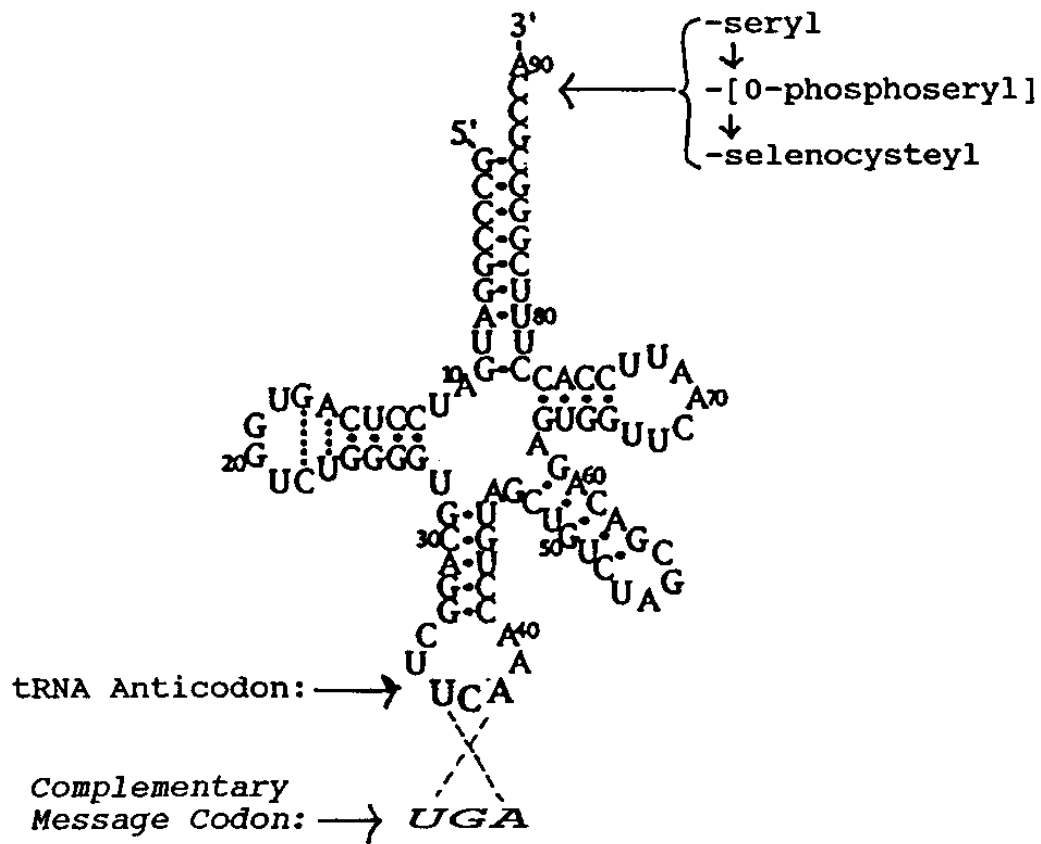


Figure 1: Nucleotide sequence of tRNA^{Sec} from a eukaryotic source. Bovine liver tRNA^{Sec} (Hatfield D., 1985) containing an esterified serine is converted to selenocysteyl-tRNA^{Sec} (Lee B.J. et al., 1989).

Similar to the bacterial system, the coordination of the synthesis of specific selenocysteine-charged tRNA, elongation factors, and a consensus sequence allows tight post-transcriptional regulation of selenoprotein biosynthesis by Se in mammals (Flohe L. et al., 1997). The consensus sequence in a stem-loop RNA structure is located in the 3' untranslated region (UTR) of the mRNA and known as a SECIS element for SeCys

insertion sequence (Kryukov, G. V., 1999). This regulation and specificity is needed in all biological systems that express selenoproteins in order to prevent read-through of normal stop codons and non-specific incorporation of selenium into sulfur-containing amino acids and enzyme cofactors.

1.3- Selenium deficiency

In mammals, when the Se supply is limited, the selenoproteins are individually synthesized based on their most essential functions (Behne, D., Kyriakopoulos, A., 1993). Several studies demonstrated that the mRNA levels of the selenoproteins fluctuate differently depending on the tissue concerned. In fact, Bermano et al. showed that with H4 hepatoma cells (Bermano G. et al., 1996), as with H3B and HL-60 myeloid cells (Baker, R.D. et al., 1993, Chada, S. et al., 1989), culture in medium without a supplement of sodium selenite led to the cells becoming Se-depleted. After 3 days in such medium the H4 cells had lost 80% of their cGpx activity. This loss of cGpx activity was accompanied by a reduction in cGpx mRNA and a smaller reduction in PHGpx activity, although there was no change in PHGpx mRNA abundance (Bermano G. et al., 1996).

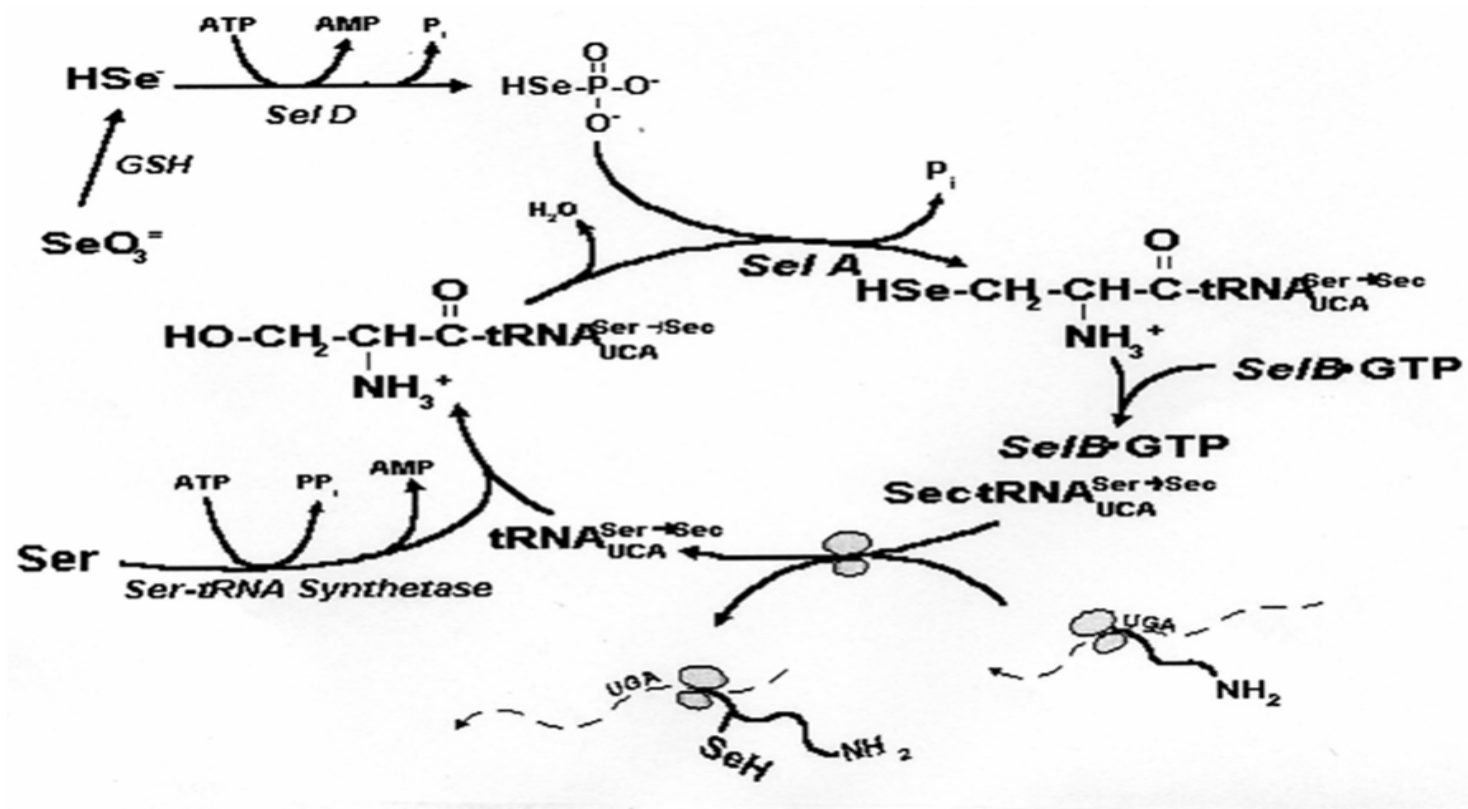


Figure 2: Overview of Selenoprotein biosynthesis (*E. coli* as a model)

Selenium deficiency causes a decrease in the concentrations of most selenoproteins but selenoprotein P and type I iodothyronine 5'-deiodinase (5'-deiodinase) are more resistant to this effect than is glutathione peroxidase. It has been shown that levels of all 3 mRNAs encoding deiodinase, selenoprotein P and Gpx decreases progressively with time by feeding a selenium-deficient diet to weanling rats for 14.5 weeks. In these experiments, mRNA decreases was correlated to decreases in Gpx activity and selenoprotein P concentration. These results suggest that synthesis of Gpx and selenoprotein P is limited to a similar extent at the translational level by the availability of selenium (Hill, K E. et al. 1992).

1.4- Selenoproteins with known function

1.4.1- Thioredoxin reductase (TrxR)

TrxR catalyzes the NADPH-dependent reduction of oxidized thioredoxin. Reduced thioredoxin provides reducing equivalents for processes such as reduction of ribonucleotides to deoxyribonucleotides by ribonucleotide reductase (Holmgren, A., 1989) and is also considered a regulator of cellular redox balance (Follmann, H., Haberlein, I., 1996), (Powis, G. et al., 1994). TrxR has been the focus of investigations for several decades but was first identified as a selenoprotein by Tamura and Stadtman in 1996 (Tamura, T, Stadtman, T.C., 1996). TrxR is a FAD-containing disulfide reductase

that plays an important role in cell proliferation (Holmgren, A., 1985). The important role of the pool of reduced thioredoxin in DNA synthesis and gene transcription implicates TrxR as a key enzyme in the control of cell growth. Mammalian forms of TrxR are not well characterized but rat liver TrxR has been previously isolated as a 116 kDa homodimer. (Luthman, M., Holmgren, A., 1982). The larger mammalian enzyme is a selenoprotein containing a SeCys residue in the sequence Cys-SeCys-Gly at the C-terminus of each subunit. (Tamura, T., Stadtman, T.C., 1996, Gladyshev, V.N. et al., 1996, Liu S.Y. Stadtman, T.C., 1997, Gasdaska, P.Y. et al., 1995).

1.4.2- Glutathione peroxidase (Gpx)

Gpx was the first mammalian selenoprotein identified (Flohe, L. et al., 1973), and as such is the best characterized mammalian selenoenzyme. In fact, the family of selenium-dependent Gpx comprises several isoforms of Gpx with differing specificity and expression patterns in different tissues that have been uncovered in recent years. The four distinct isoforms are: 1) a classic tetrameric cytosolic cGpx which is the most ubiquitous; 2) gastrointestinal cytosolic isozyme GIGpx; 3) a secreted form plasma isozyme pGpx which is released from the kidney into the plasma; and 4) the monomeric membrane associated phospholipid hydroperoxide PHGpx preferentially expressed in testis (Brigelius-Flohe, R., 1999). Each of these enzymes catalyzes the thiol-dependent reduction of hydroperoxides to their corresponding alcohols. The former three are involved in the neutralization of hydrogen peroxide (H_2O_2) via the regeneration of

glutathione (GSH), their main substrate, with the latter acting on phospholipid hydroperoxides. Mammalian glutathione peroxidases in the reduced form contain an ionized selenol that can react with an organic peroxide or H_2O_2 , to form an enzyme-selenenic acid (RSeOH) intermediate. PHGpx neutralizes oxidized membrane lipids, has a broader specificity for cholesterol and phospholipid hydroperoxides, and has been recently shown to regulate the activity of lipoxygenases (Straif, D. et al., 2000, Werz, O., Steinhilber, D., 1996, Ursini, F. et al., 1999, Flohe, L., 1989, Ursini, F. et al., 1995).

1.4.3- Methionine sulfoxide reductase (Msr)

A new selenoprotein identified both by radioisotope labeling and bioinformatic techniques (Kryukov, G.V. et al., 1999) has been shown to be a form of methionine sulfoxide reductase (Kryukov, G.V. et al., 2002) and has been termed SelR. Msr proteins convert free methionine sulfoxide and methionine sulfoxide residues in oxidatively modified proteins back to methionine in a thioredoxin-dependent manner (Brot, N. et al., 1982, Lowther, W.T. et al., 2002, Moskovitz, J. et al., 2002). Recent knockout and expression studies looking solely at the non-selenium dependent forms of these enzymes underline the important role these enzymes play in aging and oxidative stress. Studies have shown that the cyclic interconversion of Met and MetO residues of proteins is involved in several different biological processes such as: 1) an antioxidant mechanism for the scavenging of ROS; 2) the regulation of enzyme activities; and 3) cell signaling (Stadtman, E.R. et al., 2003). In the same study, they also postulated that a decrease in

Msr activities in brain tissues may be associated with the development of Alzheimer's disease.

1.4.4- Selenophosphate synthetase (SPS)

E. coli SPS is a 37 kDa enzyme that catalyzes the formation of selenophosphate (SePO_3^-), a reactive selenium (Se) donor compound from ATP and selenide and releases the beta-phosphate as orthophosphate (Leinfelder, W. et al., 1990). SPS has been isolated from an overproducing *E. coli* strain and purified (Veres, Z. et al., 1994, Lacourciere G.M., Stadtman, T., 1999). SPS uses a reduced form of selenium (NaHSe in the in vitro assay) and ATP to generate selenophosphate. From the gene sequence, *E. coli* SelD contains 7 cysteine residues, 2 of which are located at positions **17** and **19** in the sequence **-Gly-Ala-Cys-Gly-Cys-Lys-Ile-** (Leinfelder, W. et al., 1990). Mutation in SelD gene eliminates the ability of *E. coli* to incorporate selenium into selenoprotein. Inactivation of the SPS enzyme by alkylation with iodoacetamide indicated that at least one cysteine residue in the protein is essential for enzyme activity. Based on DNA sequence, a selenocysteine residue occurs in some eukaryotes and prokaryotes and in at least one of the human forms (SPS2) (Figure 3).

Organism	Conserved N-terminal domain sequence
<i>Escherichia coli</i>	LTQYSHGAG CGCK ISPKVL
<i>Salmonella typhimurium</i>	LTQYSHGAG CGCK ISPKVL
<i>Yersinia pestis</i> C092	LTQYSHGAG CGCK ISPKVL
<i>Haemophilus influenzae</i>	LTQYSHGAG UGCK ISPKVL
Homo Sapiens SPS2	LTGFSGMKG UGCK VPQEAL
Homo Sapiens SPS1	LTRFTELKG TGCK VPQDVL
Mus musculus SPS2	LTSFSGMKG UGCK VPQETL
Mus musculus SPS1	LTRFTELKG TGCK VPQDVL

U = selenocysteine

Sequences were derived from DNA sequences in the Genbank database
(NCBI, NIH)

Figure 3: Conserved Proximal dithiol/selenol motif in primary sequence of selected bacterial and mammalian SPS

This SeCys residue is located proximal to a cysteine in a conserved motif (UXC) within the N-terminal region of SPS and corresponds to a required cysteine residue in the *E. coli* enzyme, based on site-directed mutagenesis (Kim, I.Y. et al., 1992).

In these mutagenesis studies, these cysteine residues were mutated to serine residues. The authors tested the biological activities of the wild type and mutant proteins using *E. coli* MBO8 (*selD*⁻) transformed with plasmids containing the wild type and mutant *selD* genes. Their results showed that the plasmid containing the Cys¹⁷-mutated gene failed to complement the *selD*⁻ mutation in MBO8, whereas the Cys¹⁹-mutated gene was indistinguishable from wild type. The mutant proteins, like the wild type enzyme, bound to an ATP-agarose matrix, demonstrating that their affinities for ATP were unimpaired. Selenide-dependent formation of AMP from ATP was abolished by mutation of Cys¹⁷, but the Cys¹⁹ mutation had no effect on the ability of the enzyme to catalyze the reaction. These mutagenesis studies indicated that Cys17 has an essential role in the catalytic process that leads to the formation of selenophosphate from ATP and selenide.

Analysis of the total genomic sequences of *Methanococcus jannaschii* and *Haemophilus influenzae* (Bult, C.J. et al., 1996), (Fleishmann, R.D. et al., 1995) revealed that the *selD* gene in these organisms possesses a TGA codon at the position of the *E. coli* Cys-17, indicating the presence of a SeCys residue. Moreover, SeCys-containing variants also were identified in mouse and human enzymes (Guimaraes, M.J. et al., 1996), (Wilting, R. et al., 1997). Replacement of the SeCys residue of the mouse enzyme with cysteine decreased but did not abolish enzyme activity (Kim, I.Y. et al., 1997), whereas changing Cys-17 of *SelD* to Ser destroyed catalytic activity (Kim, I.Y., 1992). The second group of *SelD* homologs present in human, mouse, and *Drosophila* share high sequence similarity with the bacterial SPS but lack a cysteine or SeCys residue in the position that corresponds to Cys-17 of the *E. coli* enzyme. The human homolog has a threonine substitution, and a homolog from *Drosophila melanogaster* contains an

arginine (Low, S.C. et al., 1995) (Persson, B.C. et al., 1997). However the role of selenocysteine in the human SPS2 enzyme catalysis is still not known.

Marla Berry and collaborators (Low, S.C. et al., 1995, Low, S.C. et al., 1995) cloned and sequenced mouse and human genes exhibiting regions of homology with the *E. coli* selenophosphate synthetase (*selD*) gene. The human gene sequence encoded a motif GTGCK (residues 28-32) resembling the *E. coli* 16-20 residue segment except that Cys-17 was replaced with threonine. Expression of the human gene was shown to regulate the synthesis of ⁷⁵Se-labeled 5' deiodinase, providing evidence of the production of selenophosphate by human SPS1. Especially interesting is the finding that an analogous gene detected in the mouse embryo at early stages of development contains an N-terminal sequence region corresponding to the *E. coli* residue 16-20 segment except that a selenocysteine residue is encoded in place of the essential Cys-17 residue as shown in figure 3 (Zlotnik, A. et al., 1995, Guimaraes, M.J. et al., 1995).

SPS represents the first defined step in the specific incorporation of selenocysteine into selenoproteins, since the transport and reduction of selenium has not been defined at the molecular level. Although trafficking of selenium to SPS is poorly understood, recent evidence indicates that a selenium binding protein (Self, W.T. et al, 2004) may be involved in linking transport and reduction of Se to SPS in a bacterial model system. Previous kinetic studies under strictly anaerobic conditions reported an apparent *K_m* of 7.3μM for sodium hydrogen selenide (NaHSe) using *E.coli* SPS (Veres, Z., 1994). The authors measured the formation of AMP from ATP after separation ¹⁴C-labeled nucleotides by thin layer chromatography (TLC). Although NaHSe is the only known substrate for SPS, it is likely that physiological concentrations of NaHSe do not

exceed nanomolar levels. Thus, the trafficking of Se to SPS is still a key issue to be resolved in Se metabolism.

1.5- Arsenic as a carcinogenic metalloid

Industries release quantities of numerous metals and metalloids such as arsenic (As) into the environment exposing humans to elevated concentrations of these toxic elements. Inorganic As is also naturally occurring, ubiquitous in the environment in the form of trivalent (AsIII) as well as pentavalent (AsV) species (Hei, T.K., Filipic, M., 2004). Unlike selenium, arsenic has no known role in biology. In contrast to selenium, arsenic has been implicated as a contributing factor in carcinogenesis (Hughes, M. F., 2002). Biologically, the trivalent sodium arsenite is more toxic than the pentavalent sodium arsenate (Barrett, J.C. et al., 1989). Chronic exposure to arsenic is associated with skin, lung, liver and bladder cancers with skin being the major target, (Germolec, D.R. et al., 1998, Landsdown, A.B., 1995, Phillip, R., 1985), however the biochemical mechanism of this arsenic-induced carcinogenesis is not well described.

Cultured mammalian cells have provided some possible mechanisms by which arsenical compounds may exert a carcinogenic activity. Sodium arsenite and sodium arsenate were observed to induce morphological transformation such as enhanced colony-forming efficiency of Syrian hamster embryo cells in a dose-dependent manner (Lee, T.C. et al., 1985). The trivalent sodium arsenite was more potent sulfhydryl depleting agent by oxidation of GSH to GSSG than the pentavalent sodium arsenate (Winski, S.L.,

Carter, D.E., 1995). A recent study has demonstrated that arsenite treatment of cultured cells results in the production of reactive oxygen species and a prolonged oxidative stress correlated with a consistently high level of Heme oxygenase I mRNA (Gabis, K.K. et al., 1996, Takahashi, S. et al., 1998). DNA microarray studies demonstrated early upregulation of three different TrxR genes following arsenite treatment (Hamadeh, H.K. et al., 2002, Rea, M.A. et al., 2003). It is clear from these studies that treatment of cultured mammalian cells results in oxidative stress, yet the molecular mechanism behind this oxidative stress is unknown.

1.6- Arsenite interactions with glutathione

Arsenite, the predominant trivalent species of arsenic in the environment, is known to react with intracellular thiols such as glutathione (GSH) (Scott, N. et al., 1993). Reactions between GSH and arsenite have been characterized using ^1H and ^{13}C NMR. These investigators followed binding of As to GSH through the thiol group by shifts in the carbon atom bonded to the sulfhydryl group of the cysteinyl residue, i.e., the CH_2 carbon atom and the protons bonded to it. Sodium arsenite, As(III), bound to GSH to form an $\text{As}(\text{SG})_3$ complex in solution as indicated by NMR spectra. The toxicity of arsenite has been attributed to the inhibition of enzymes with proximal or vicinal thiol residues, such as the lipoamide-dependent α keto-acid dehydrogenases of the Krebs cycle (Lin, S. et al., 2001, Gorin, Y., Leseney, A.M., 1997, Cavigelli, M. et al., 1996). More recently, TrxR has also been shown to be inhibited by trivalent arsenic compounds both

in vitro and *in vivo* (Lin, S. et al., 1999, Styblo, M., Thomas, D. J., 1995). However, 50% inhibition required greater than 100 μ M arsenite. Other members of the pyridine-nucleotide dependent family of oxidoreductases, such as GSH reductase, have also been shown to be inhibited by arsenite and other trivalent arsenic species (Styblo, M., Thomas, D. J., 1995). Each of the pyridine-nucleotide dependent oxidoreductases has conserved proximal cysteines (and/or selenocysteines) within their active sites. It is presumed that arsenite is reacting with and/or binding to these sites in the inhibition of these enzymes, although direct binding studies have not been performed. Arsenite has high affinity to GSH and free radicals induced by arsenite can oxidize GSH, hence depleting the level of antioxidant. Arsenic-thiol interactions were investigated by determining changes in rat blood sulfhydryls after exposure to arsenate, As(V), or arsenite, As(III). (Winski, S.L., Carter, D.E., 1995).

1.7- As/Se mutual sparing effect

The first experimental evidence indicating a link between the metabolism of inorganic arsenic and selenium was reported more than 60 years ago (Moxon, A. L., 1938) after feeding rats with sodium arsenite (in drinking water) along with seleniferous grains in order to prevent Se poisoning. In these studies, the poisonous arsenite was capable of preventing toxic effect of selenium, thus, these metalloid displayed a mutual sparing effect. Later studies demonstrated that the chronic ingestion of arsenite abolishes the anticarcinogenic effect of Se in rats (Ip, C.; Ganther, H. E. 1988), providing a

potential explanation of the association of cancer with prolonged As intake in humans (Chen, C.J. et al.; 1988, Wu, M.M. et al., 1989). In fact, several studies showed the link between high As/Se ratio (lung, blood) and carcinogenesis. (Wester, P.O. et al., 1981; Wang, C.T. 1996). Research on Blackfoot disease patients proved As to be the major causative agent of the disease and the authors attributed the decrease of Se to the antagonistic effect of arsenic (Wang, C.T. et al., 1993). Blackfoot disease (BFD) is a severe form of peripheral vascular disease, in which the blood vessels in the lower limbs are severely damaged, resulting eventually in progressive gangrene. This disease has been limited to certain areas of Taiwan with elevated levels of arsenic.

More recent studies on residents living in an As-contaminated area in Bangladesh corroborated the previous observation by suggesting that As alters Se metabolism, increasing Se excretion through bile or feces (Miyazaki, K. et al., 2003).

1.8- Arsenic and Selenium containing thiol conjugates

Previous studies have shown seleno-bis (S-glutathionyl) arsinium ion $[(GS)_2AsSe]^+$ to be the major As and Se excretory product in bile of rabbits treated with arsenite and selenite (Gailer, J. et al., 2002, Gailer, J., 2002). In these studies, the authors have intravenously injected rabbits with different combinations of the arsenic and selenium oxo-anions (arsenite + selenate, arsenate + selenite, and arsenate + selenate) and analyzed the collected bile and whole blood samples by X-ray absorption spectroscopy.

Only the injection of arsenite and selenate led to the biliary excretion of $[(GS)_2AsSe]^-$ within 25 min. In these studies, the stability of the compound was not addressed.

The antagonism between arsenite and selenite may have its molecular basis in the formation of the arsenic-selenium compound. Previous work on the metabolism of arsenate(AsV) and selenate in mammals (mice and rabbits) by determination of arsenic metabolites in plasma and urine after administration of $[^{74}As]$ arsenate revealed that these compounds are individually reduced in vivo to arsenite (AsIII) (Vahter, M., Envall, J., 1983, Vahter, M., Marafante, E. 1985) and selenite (Kobayashi, Y. et al., 2001). When As and Se are co-administered, a mutual inhibition of the individual methylation pathways occurs and this may be explained by the formation of $[(GS)_2AsSe]^-$ (Levander, O. A.; Argrett, L. C., 1969; Hsieh, H. S.; Ganther, H. E., 1975; Zakharyan, R. et al.; 1995). The biliary excretion of $[(GS)_2AsSe]^-$ may be especially important in view of the fact that prolonged exposure to inorganic arsenic in drinking water significantly reduces tissue selenium concentrations (Wang, C.T., 1996). Discovery of the As-Se complex may be used against the pathological effects of inorganic arsenic in drinking water (in Bangladesh for example) by increasing the daily intake of selenium leading to the formation and excretion of $[(GS)_2AsSe]^-$.

Based on known interactions of As with vicinal dithiols and the fact that selenophosphate synthetase also contains a proximal cysteine motif (Figure 3), we have tested the possibility that arsenite and/or $(GS)_2AsOH$ (AsIII) would inhibit SPS. In order to facilitate this kinetic analysis, we have developed a spectrophotometric assay to analyze the initial rate of selenide-dependent production of inorganic phosphate (Figure 5) from *E. coli* SPS. We also determined whether As treatment of cultured cells

(Hela and HaCaT keratinocyte) would have an effect on the incorporation of Se into selenoproteins using the radioisotope ^{75}Se . A better understanding of the interrelationship of arsenic and selenium could lead to a possible mechanism of arsenic-induced carcinogenicity that may open a door towards an efficient treatment of population exposed to high levels of arsenic.

CHAPTER TWO: MATERIALS AND METHODS

2.1- Selenophosphate synthetase spectrophotometric assay

2.1.1- Assay Principle

A coupled assay for the determination of inorganic phosphate (Pi) described by Webb (Webb, M.R., 1992) was used to follow NaHSe-dependent SPS activity. This sensitive enzymatic assay was used to monitor the kinetics of phosphate release by SPS in solution through formation of a chromophoric product. In the presence of inorganic phosphate (Pi), 2-amino-6-mercapto-7-methylpurine riboside (MESG) is converted by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine.

An increase in absorbance at 365 nm is monitored spectrophotometrically and proportional to Pi consumption by the MESG/PNP reaction (Figure 4).

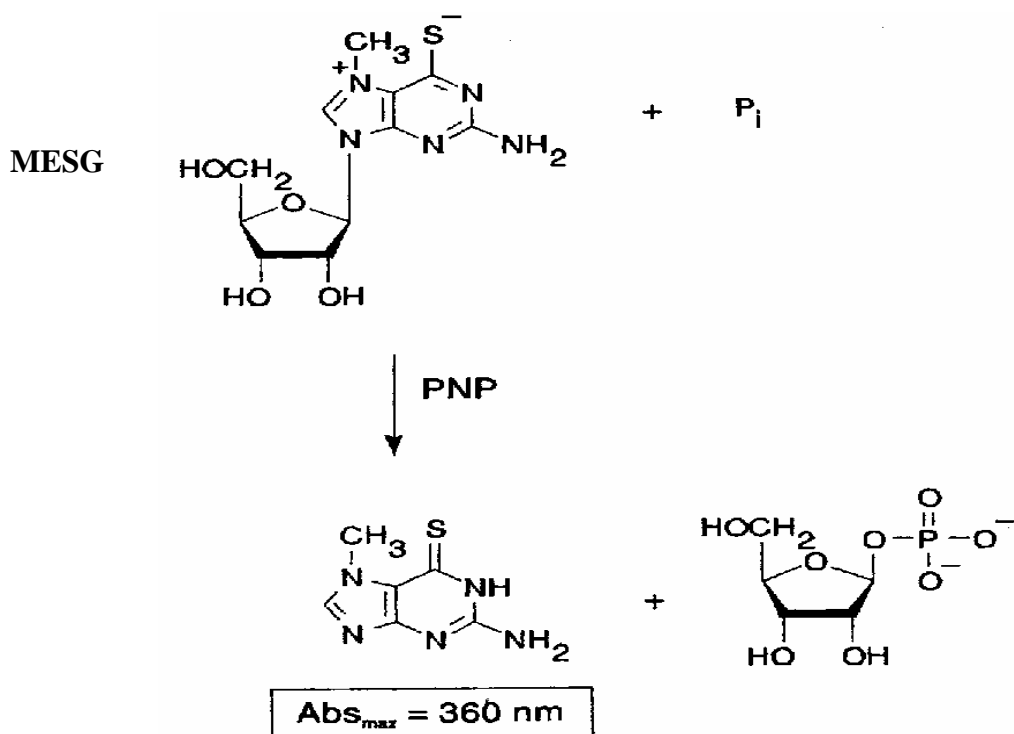


Figure 4: Enzymatic conversion of 2-amino-6-mercapto-7-methylpurine riboside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP). The accompanying change in absorption at 365 nm allows quantitation of inorganic phosphate (Pi) consumed in the reaction.

2.1.2- Synthesis of sodium hydrogen selenide

NaHSe was synthesized as previously described (Klayman, D.L., Griffin, T.S., 1972). The reagents were purchased from Acros Organics. All the reactions were performed in an anaerobic chamber (Coy Laboratory Products, Inc. Grass Lake,

Michigan). Equation (1) summarizes the reaction of sodium borohydride with elemental Se to yield sodium hydrogen selenide.



Aqueous solution of sodium borohydride (1.5 mM) was added drop wise at room temperature to an aqueous solution of metallic selenium (0.725 mM) in a 2:1 molar ratio. The reaction was completed within 15 minutes and colorless NaHSe resulted (590 mM final concentration). NaHSe was separated from residual borate by passage through a G10 column (30 X 1 cm from Sigma-Aldrich) and stored at room temperature under anaerobic conditions until used as a selenium substrate for SPS assays.

2.1.3- Spectrophotometric assay procedure

The assay was performed at 37 °C in an anaerobic cuvette (Fisher Scientific) due to oxygen sensitivity of the NaHSe and the product selenophosphate according to equation (Figure 5) (Bock, A., Stadtman, T.C., 1988; Kramer, G.F., Ames, B.N. 1988; Wittwer, A.J., Stadtman, T.C. 1986).



Figure 5: Overall Reaction catalyzed by SPS

The reaction buffer included 100 mM Tris (ICN Biomedicals), 20 mM KCl (Fisher Scientific), 4 mM MgCl₂ (Alfa Aesar) and the pH was adjusted to 7.2. All the reagents were degassed in the anaerobic chamber prior to use. The 700 µl reaction mixture contained: 0.2 mM MESG substrate, 1 U of purine nucleoside phosphorylase (PNP) (Molecular Probes), 2 mM ATP (Alexis Biochemicals), variable concentrations of *E. coli* SPS and NaHSe. The reaction mixture was equilibrated to 37 °C in a sealed anaerobic cuvette (Fisher Scientific) using a thermostatable cell holder incorporated in the Agilent 8453 UV-Visible spectroscopy system (Alpha-Omega Technologies, Inc., Brielle, New Jersey). A uniform temperature was maintained by circulating water from a thermostatted water bath through the manifold. The spectrophotometer was first blanked using previously cited mixture without ATP and NaHSe. A baseline activity reading was taken prior to the addition of ATP using a gas-tight syringe. The NaHSe-dependent production of Pi was measured by ascertaining the increase in absorbance at 365 nm as previously described (Figure 6). For SPS inhibition studies, sodium arsenite was diluted for each experiment from a 100 mM stock solution and different concentrations (10, 17.5 and 25 µM) added anaerobically before the substrate selenide which was incorporated last to the reaction cuvette.

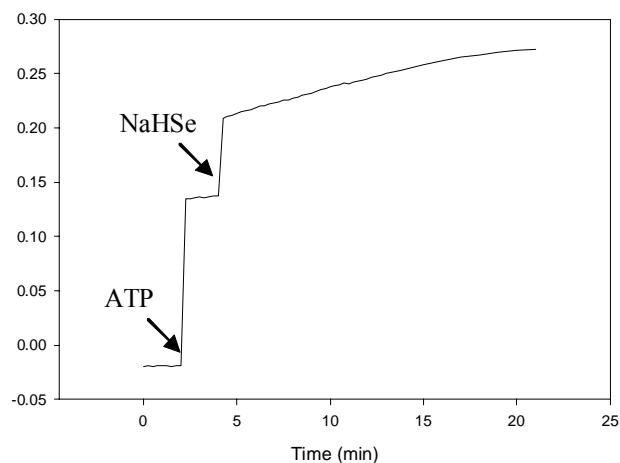


Figure 6: Representative SPS Assay: NaHSe-dependent production of Pi. The arrows indicate the times of addition of the reagents.

2.1.4- Synthesis of Seleno-bis (S-glutathionyl) arsinium ion [(GS)₂AsSe]⁺

All the reactions and purification were performed under anaerobic conditions at room temperature. NaHSe was previously made for SPS assay.

- $\text{As}(\text{OH})_3 + 2\text{GSH} \longrightarrow (\text{GS})_2\text{AsOH} + 2\text{H}_2\text{O}$
- $(\text{GS})_2\text{AsOH} + \text{NaHSe} \longrightarrow [(\text{GS})_2\text{AsSe}]^+ + 2\text{H}_2\text{O}$

(GS)₂AsOH (0.1 mM) was prepared by mixing aqueous GSH (0.2 M) and sodium arsenite solution (0.1 mM) in 2:1 molar ratio and the pH adjusted to 7.6. NaHSe and

(GS)₂AsOH were mixed in a 1:1 (V/V) ratio to bring the final pH to 8.5. The compound was purified by passage through G10 column (30 cm-column) under strictly anaerobic conditions. An aliquot of each fraction was exposed to the ambient air to allow the detection of Se-containing eluates. Pure [(GS)₂AsSe]⁻ was recovered by pooling the Se-containing fractions after gel filtration. (Gailer, J. et al., 2002)

2.2- Cultivation of Keratinocytes

2.2.1- Culture

A keratinocyte cell line, HaCaT (Kind gift of Dr. Norbert Fusenig, Institute of Biochemistry, Cancer Research Center, Heidelberg, Germany), was cultured in Dulbecco's modification of Eagle's medium 1X (DMEM) with L-Glutamine and 4.5 g/l glucose supplemented with 10 % fetal bovine serum (FBS), 10,000 µg/ml streptomycin, and 10,000 IU/ml penicillin (Mediatech, Inc.). The cells were incubated at 37 °C with 5 % carbon dioxide to maintain proper pH. The culture was collected treating the cells with 0.05 % EDTA- Dulbecco's Phosphate Buffered Saline (DPBS) without calcium/magnesium (Ca/Mg), then trypsin and transferring into a centrifuge tube as previously described (Fusenig, N.E., 1971).

2.2.2- Culture in basal medium

Due to the presence of selenoprotein P in the serum along with undefined forms of Se, we also cultured the keratinocytes in defined keratinocyte medium with L-Glutamine and growth supplement (Gibco, Grand Island, New York). These cultures were routinely acclimated to the defined medium for at least one passage before each experiment.

2.2.3- Long term arsenite treatment

At 3-4 days post-confluence, the cells were split at a 1:5 ratio and the medium changed the next day. To the fresh medium was added 0.5 and 1 μ M sodium arsenite (in triplicate) along with untreated control cultures. Images of the cultures were taken daily using an inverted microscope to document any morphology changes. The cells were then washed with DMEM then DPBS with Ca/Mg by centrifugation and the cell pellet was resuspended in lysis buffer for further sonication. Lysis buffer contained 50mM Tricine pH 8.0, 0.1 mM Benzamidine, 0.5mM EDTA and 1mM DTT.

The crude cell lysate was clarified by centrifugation at 13000 x g for 10 minutes. This crude cell extract was used for further determination of enzyme activities or immunoblot analyses of selenoproteins.

2.2.4- Short term arsenite treatment

At confluency, the cells were treated with 2 μ M sodium arsenite for 4 days. Untreated cells served as controls. Every 24 hours, control and treated cells were harvested as described in the previous section and the cell pellet stored at -20 °C until needed for further analysis.

2.2.5- Short term arsenite treatment of cells grown in defined medium

To determine the involvement of other selenoproteins in the FBS as Se source, the cells were grown in defined keratinocyte medium (Basal medium) for 72 hours, split (1:2), then treated the next day with 2 and 4 μ M sodium arsenite for another 72-hour period. Every 24 hours, a subset of cultures were harvested and stored at -20°C for further analysis.

2.2.6- MTT Cell Proliferation Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay provides a simple method for determination of cell number using standard microplate absorbance reader. This assay was developed by Mossman (Mossman, T., 1983) and involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide) to an insoluble formazan (Liu, Y. et al., 1997; Berridge, M.V., Tan, A.S. 1993; Vistica, D.T. et al., 1991). The formazan is then solubilized, and the concentration determined by optical density at 570 nm.

Assay Protocol

HaCaT cells were cultured in 96-well plates (0.31 cm² growth surface, 340 µl volume) in defined medium which contains 10 nM sodium selenite (ICN Biomedicals, Inc., Aurora, OH) for 24 hours and treated with increasing concentrations of sodium arsenite (0, 3, 6, 10, 12, 14 and 16 µM in triplicates) for another 24-hour period. A subset of cultures was supplemented with 40 nM and 90 nM sodium selenite to reach a final concentration of 50 and 100 nM.

A 12 mM MTT (Amresco, Solon, OH) stock solution in sterile PBS pH 7.4 was made and stored at 4°C protected from light (for four weeks maximum). MTT was added (10 µl) to each well (96-well plate) to obtain a final concentration of 1.2 mM. A negative control of 10 µl of the MTT stock solution added to 100 µl of medium alone was included. The culture was then incubated at 37 °C for 2-4 hours. After incubation of the cells with MTT, as described above, a solution of 10 % SDS- 0.01 M HCl (100 µl) was added to each well as a solubilizing agent to dissolve the formazan (Carmichael, J. et al., 1987) and mixed thoroughly with the pipette. The culture was then incubated at 37 °C for 18 hours. The samples were mixed again and absorbance read at 570 nm using a SpectraMax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA) or Dynex MR (DYNEX Technologies, Inc., Chantilly, VA).

2.3- Bradford Assay

2.3.1- Principle

The Bradford Assay was used for determining protein content of cell extracts or purified protein concentrations preparations. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change (Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248).

Bradford reagent: Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95 % ethanol, added 100 ml 85 % (w/v) phosphoric acid. The solution was diluted to 1 liter when the dye completely dissolved, and filtered through Whatman #1 paper. A standard curve of absorbance at 595 nm versus protein concentration was routinely prepared for protein assays using BSA as a standard.

The spectrophotometer was warmed up for 15 min before use and the samples diluted with distilled water to an estimated concentration of 1 to 20 µg/ml. Standards containing a range of 1 to 2.5 µg protein (bovine serum albumin) to a volume of 200 µl were prepared. Dye reagent (1 ml) was added and incubated 5 min. Absorbance at 595 nm was measured.

2.4- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyze the protein content of cell lysates and as the first step for immunoblot analysis of selenoenzymes. A 12 % polyacrylamide gel was used to resolve the proteins and 5 % gel to stack the extract in the lanes. The 4X separating gel buffer consists of 0.4 % SDS, 1.5 M Tris-HCl, pH 8.8. The 4X stacking gel buffer consists of 0.4 % SDS, 0.5 M Tris-HCl, pH 6.8. Our 10ml separating gel contained 2.5 ml 4X separating buffer, 4.0ml 30 % acrylamide stock solution (29.2 % acrylamide, 3.5 ml distilled water, 100 μ l 10 % ammonium persulfate, and 10 μ l TEMED. The 5 ml stacking gel contained 1.25 ml 4X stacking buffer, 0.83 ml (4 %) 30 % acrylamide stock solution, 2.9 ml distilled water, 50 μ l 10 % ammonium persulfate and 5 μ l TEMED. The running buffer contained 25 mM Tris-HCl, 192 mM Glycine and 0.1 % w/v SDS. The samples were denatured by addition of 2X SDS-PAGE sample buffer (4X stacking gel buffer, 20 % glycerol, 4 % SDS, 31 mg/ml DTT) and the samples were boiled for 5 minutes, followed by a 10-minute cooling at room temperature. Proteins were subjected to electrophoresis for 55 minutes at 150 volts.

2.5- Western blot analysis

Western blot were used to analyze the level of glutathione peroxidase (Gpx) and thioredoxin reductase (TrxR) proteins in the control and arsenite-treated samples. Thirty to 50 μ g of protein extract (varied in alternate experiments) was analyzed using 12 % SDS-PAGE, and subsequently transferred (transfer buffer 25 mM Tris-HCl, 192 mM

Glycine, 20 % v/v Methanol) onto a hydrophobic polyvinylidene fluoride (PVDF) membrane. The transfers were routinely performed overnight at 30 volts for optimal transfer of protein. The membrane was incubated in a 2 % bovine serum albumin solution (BSA) in TBS-Tween for 1 hour to block the membrane. The primary antibodies used at 1/1000 dilution were polyclonal sheep anti-Gpx (company) or rabbit anti-TrxR (Stadtman, T.C., NIH). The secondary antibodies (1/1000 dilution) were rabbit anti-sheep, and goat anti-rabbit each conjugated with alkaline phosphatase. The required reagents were Tris buffered saline with Tween-20 (TBS-T) 20 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.01 % Tween-20), 2 % BSA in TBS-T and phosphatase alkaline buffer (0.1 M Tris, 100 mM NaCl, 5 mM MgCl pH 9.5). We used a chromogenic detection system with nitro blue tetrazolium (NBT, 5mg/ml of 70 % Dimethyl Formamide, DMF) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 5 mg/ml of 100 % DMF) in alkaline phosphatase buffer to visualize the presence of bound secondary antibody.

2.6- Selenium (^{75}Se) labeling of selenoproteins

HaCaT cells were cultured in DMEM with 10 % FBS in the presence of arsenite (200 nM to 8 μM). The keratinocytes were radiolabeled with 100 μl ^{75}Se (approximately 10 μCi) in 10 nM sodium selenite for 8 hours. Cells were then harvested, lysed by sonication, clarified by centrifugation (13,000 x g) and 28 μg of cell extract was loaded in each lane (determined by Bradford assay with BSA as standard). The gel was stained with Coomassie blue G250 for 1hour, then destained for 30-60 min

with a solution composed of 50 % methanol and 10 % acetic acid. After drying the gel overnight (DryEase Minigel Drying System from Invitrogen, Carlsbad, CA), and incubation for 3 days in the phosphor storage cassette, the radiolabeled proteins were visualized using a Storm 840 phosphorimager (Molecular Dynamics/ Amersham BioSciences). The storage cassette screen retains energy from beta particles, X-rays, and gamma rays. Upon laser-induced stimulation, light is emitted from the storage phosphor screen in proportion to the amount of radioactivity in the sample. ^{75}Se isotope incorporation was also determined using a Perkin-Elmer 1470 Gamma counter from Life Sciences and quantitatively compared to total protein concentration to determine the efficiency of Se incorporation (cpm/ μg of extract).

2.7- Sulfur (^{35}S) labeling

HaCaT keratinocytes were grown in 12-well plates (3.66 cm^2 growth surface, 6.30 ml volume), and then treated with increasing concentrations of arsenite from 200 nM to 6 μM for 4 hours. Two μl (29 μCi) of ^{35}S (Redivue Pro-Mix ^{35}S , Amersham BioSciences, San Francisco, CA) in the form of Cys and Met in 2 ml DMEM (low Cys and Met) was used to label newly synthesized proteins during arsenite treatment. After the 4-hour period, the cells were harvested, lysed and a crude cell extract made as stated previously in section 2.2.3. To determine the radioactivity (^{35}S) in each extract, 5 μl of sample was added to 3.5 ml of Opti-Fluor scintillation liquid (PerkinElmer Life Sciences, Boston, Massachusetts) and read using a TriCarb 2900TR liquid scintillation analyzer

(Packard BioScience, Boston, Massachusetts). Equal concentration of protein (1 µg) was analyzed by SDS-PAGE. The 12 % gel was then dried immediately without staining. After drying and incubation for 3 days in the phosphor storage cassette, sulfur incorporation into protein was analyzed by a phosphorimager (Amersham BioSciences).

2.8- Haemophilus influenzae (H. i.) SPS overexpression

A selenocysteine-containing SPS was overexpressed using *E. coli* BL21 strain that contained the plasmid coding for HISPS (pET3aHIselD). The plasmid was obtained as a generous gift from T.C. Stadtman (NHLBI, NIH, Bethesda, Maryland). Cells were grown aerobically in 2.8-liter flasks containing Luria broth (10 g tryptone, 5 g yeast extract, 5 g NaCl /L) supplemented with 0.3 % dextrose, 10 mg/L chloramphenicol and 100 mg/L ampicillin. Flasks were incubated at 37 °C with vigorous shaking (180 rpm) until the optical density (OD) reached 0.7 at 600 nm. Expression of HISPS was then induced with Isopropyl-β-D-thiogalactopyranoside (IPTG) and cultured at room temperature for 24 hours. OD readings and samples were taken at 2 hour intervals for 6 hours and then overnight. Cells were harvested using a SLA 1500 rotor (Sorvall) centrifuge, 5,000 rpm at 4 °C for 10 minutes. Cell pellets were washed in buffer A (50 mM Tris, 0.5 mM EDTA, 1 mM DTT), then lysed in lysis buffer (50mM Tricine pH 8, 0.1 mM Benzamidine, 0.5 mM EDTA, 1mM DTT) by sonication and the cell debris were removed by centrifugation at 13,000 rpm for 20 minutes.

2.9- Haemophilus influenzae (H. i.) SPS purification

The purification process was done in 3 steps in the following order: Phenyl Sepharose chromatography, Diethylaminoethyl Fast Flow anion-exchange chromatography (DEAE) and then gel filtration chromatography (Sephacryl S-200) (Amersham BioSciences).

Phenyl Sepharose chromatography

All the solvents and buffers were degassed prior to use. The column was prewashed with 3 column volumes distilled water, followed by passage of 2 column volumes of buffer A. The column was then equilibrated with buffer A containing 1.5 mM ammonium sulfate and the sample loaded. A 300 ml gradient of ammonium sulfate (1.5 mM-0 mM) was made with the PACE gradient maker (Bioworld, Dublin, Ohio) to elute the bound proteins. After a thorough wash with buffer A, the SPS-containing fractions were eluted with 4 column volumes of 30 % ethylene glycol (EG) in buffer A. Fractions (5 ml) were collected and the presence of HISPS was assessed by western blot using polyclonal antibody raised against the *E. coli* SPS. The fractions containing SPS were pooled concentrated down to a smaller volume, then dialyzed twice overnight (against 1L of dialysis buffer) to remove the ethylene glycol before loading on a DEAE column. The dialysis buffer contained 0.5 mM Tris pH 7.5, 0.5 mM EDTA, 0.1 mM DTT.

DEAE chromatography

The dialyzed pooled sample from phenyl chromatography was then applied to a pre-equilibrated DEAE column. A 0.0-0.5 M potassium chloride (KCl) gradient was used

to elute bound proteins. The column was washed with buffer A containing 0.5 M KCl until no significant protein eluted from the column (by Bradford assay, see section 2.3). The column was cleared of any remnant protein by passage of 2 M sodium chloride (NaCl). Fractions (5 ml each) were collected during the 0.0-0.5 M KCl gradient and the presence of Hi SPS was determined by SDS-PAGE and western blot. The fractions containing Hi SPS were pooled and concentrated down to a smaller volume. No dialysis was necessary because the salt concentration in the sample was ideal for the next step of the purification process.

Gel filtration chromatography

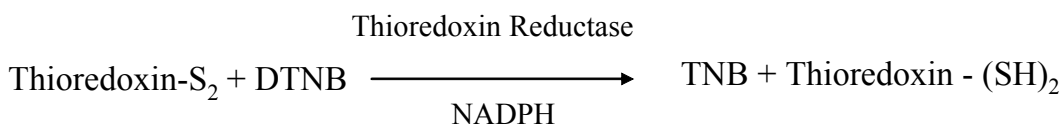
The column was slowly washed overnight with a large volume (2 liters) of distilled water and equilibrated with buffer A containing 0.3 M KCl for 6 hours. The sample was then loaded manually onto the column close to the column medium. Once the solvent entered the column, being careful not to perturb the column, the buffer was then pumped through the column at a slow rate (approximately 1.5 ml/min). Fractions were collected and the purity of HISPS assessed by SDS-PAGE. The sample (110 mg) was determined to be greater than 98 % pure based on SDS-PAGE analysis (Coomassie Blue staining). The final concentration was determined by absorbance reading at 280 nm in 6 M Guanidinium, HCl in 25 mM Tris pH 7.8 using the predicted molar extinction coefficient of $8,400 \text{ M}^{-1} \text{ cm}^{-1}$.

2.10- Sample preparation for X-ray atomic absorption (XAS)

A sample of pure HISPS was prepared for XAS analysis by overnight dialysis against 50 % glycerol in 50 mM Tris, then concentrated to 35 mg/ml protein. A 625 μ M SPS in 50 % glycerol in 50 mM Tris was sent to Dr. Robert Scott at the Department of Chemistry, University of Georgia, Athens, Georgia for Se XAS analysis.

2.11- Gpx and TrxR activity assay

2.11.1- TrxR



DTNB = 5,5'-Dithio-bis(2-Nitrobenzoic Acid)

TNB = 5-Thio-2-Nitrobenzoic Acid

NADPH = Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form

This assay is a continuous spectrophotometric rate determination. Thioredoxin reductase activity was measured as NADPH-dependent 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) reduction. The one ml assay mixture contained 100 mM potassium phosphate buffer pH 7.0, 0.2 mM NADPH, 2.5 mM DTNB and 10 mM EDTA. The spectrophotometer was blanked with the above mixture then the assay started with

addition of 200 µg of protein extract (Gorlatov, S. N., Stadtman, T. C., 1998). A spectrum was recorded every 15 seconds for 22 minutes, 2 minutes before addition of the extract and 20 minutes after to determine the baseline rate of DTNB reduction and subsequently the NADPH-dependent reduction of DTNB in the presence of cell extract.

2.11.2- Gpx

Gpx activity was determined using a previously described coupled assay with glutathione reductase with slight modifications (Lawrence, R.A., Burk, R.F., 1976). The assay buffer contained 100 mM potassium phosphate buffer pH 7.0, 0.2 mM NADPH, 0.1 mM EDTA, 1 mM GSH, 240 mU glutathione reductase, 50 µg of protein extract that was used to blank the cuvette. The oxidation of NADPH by hydroperoxides was followed spectrophotometrically at 340 nm after addition of 250 µM hydrogen peroxide (H₂O₂) to the reaction mixture. A spectrum was recorded every 15 seconds for 12 min., 2 min. before addition of H₂O₂ and 10 minutes after. Initial rate of reduction of hydroperoxides was determined by monitoring the change in absorbance at 340 nm.

CHAPTER THREE: RESULTS

3.1- Inhibition of Selenophosphate synthetase

Exposure to arsenic by ingestion of contaminated drinking water is linked to skin, lung, kidney, liver and bladder cancer. The toxicity of trivalent arsenicals such as arsenite is related to their high reactivity with vicinal dithiols. Arsenite is known to bind to vicinal sulfhydryl groups on small compounds such as glutathione (GSH). It was shown that *E. coli* SPS contains seven Cys residues and two of them are located at the amino-terminal region forming a CXC motif. Such motif with vicinal dithiols could represent a binding site for trivalent arsenicals which makes SPS an interesting selenoprotein to study. In addition, SPS is a key enzyme in the selenoprotein biosynthesis pathway. SPS catalyzes the activation of selenide into selenophosphate in the presence of ATP, the first step of the pathway.

To determine if sodium arsenite inhibits SPS activity *in vitro* with sodium selenide as a substrate, we have spectrophotometrically followed the effect of arsenite on SPS activity. The experiment was done under strictly anaerobic conditions. Arsenite does not inhibit the coupled reaction, as we have determined that up to 100 μ M arsenite does not affect PNP reaction with MESG (data not shown). Addition of arsenite resulted in the inhibition of SPS activity (Figure 7). Figure 7 illustrate that arsenite inhibits the NaHSe-dependent hydrolysis of ATP in a concentration-dependent manner.

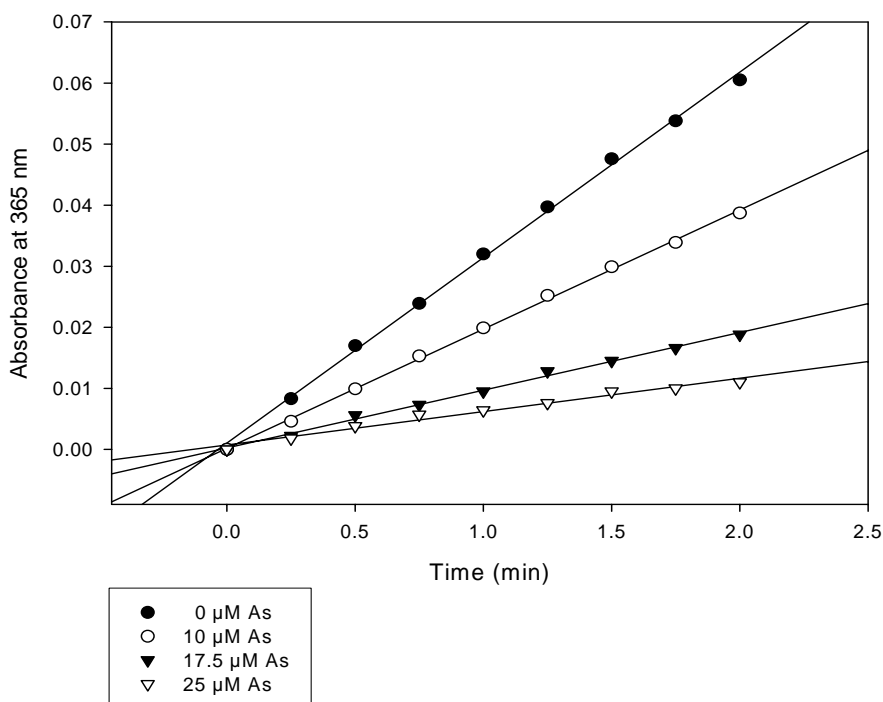


Figure 7: Initial rate plot of a typical SPS assay with 0, 10, 17.5, 25 μM arsenite. Each point represents at least three independent enzyme assays.

To determine whether this inhibition is competitive with NaHSe, we analyzed SPS activity at several concentrations of NaHSe while varying the concentration of arsenite. A summary of these activities is shown in a double-reciprocal plot of SPS activity versus [NaHSe] in figure 8. Inhibition of SPS by arsenite is competitive. A plot of the slopes of the lines in figure 8 versus arsenite concentration revealed an apparent K_i of 3 μM . This is roughly half the apparent K_m we have measured for SPS (7.8 μM), demonstrating that arsenite or other trivalent arsenicals could be relevant inhibitors *in vivo*.

Our results are consistent with previous findings (K_m of 7.3 μM for NaHSe) using ^{14}C -TLC, a different method. (Veres, Z., 1994).

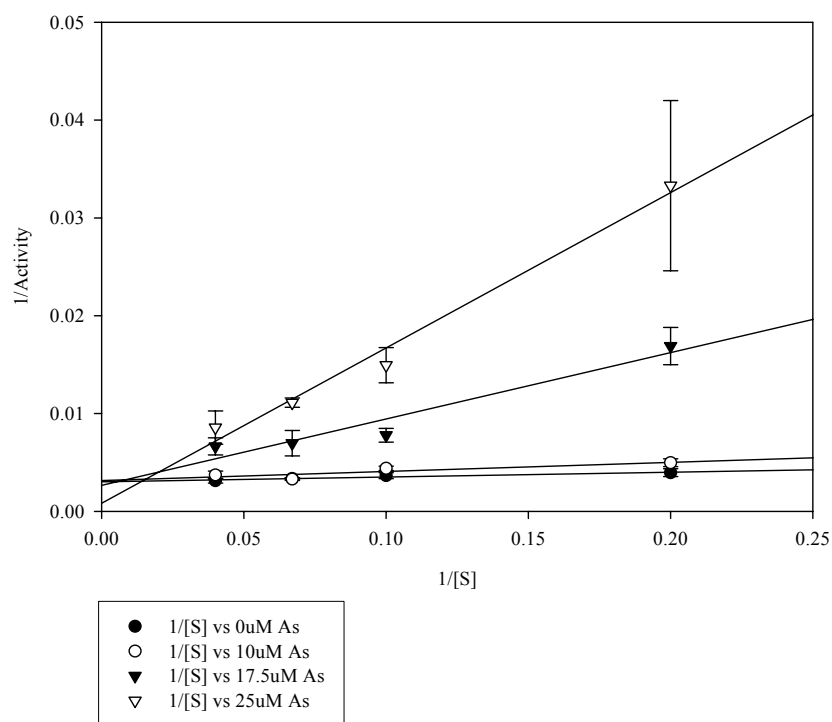


Figure 8: Double reciprocal plot to analyze the type of inhibition of SPS by arsenite. Each point represents at least three independent enzyme assays.

Arsenic binds to macromolecules such as glutathione upon entry into the cell (Winski, S.L, Carter, D.E., 1995).

Hence, we have tested the possibility that the glutathiolated derivative, $(GS)_2AsOH$ being a trivalent *in vivo* form of arsenic, acts as SPS inhibitor. As shown on figure 9 $(GS)_2AsOH$ also inhibits SPS in a concentration-dependent manner (10, 25 and 50 μM).

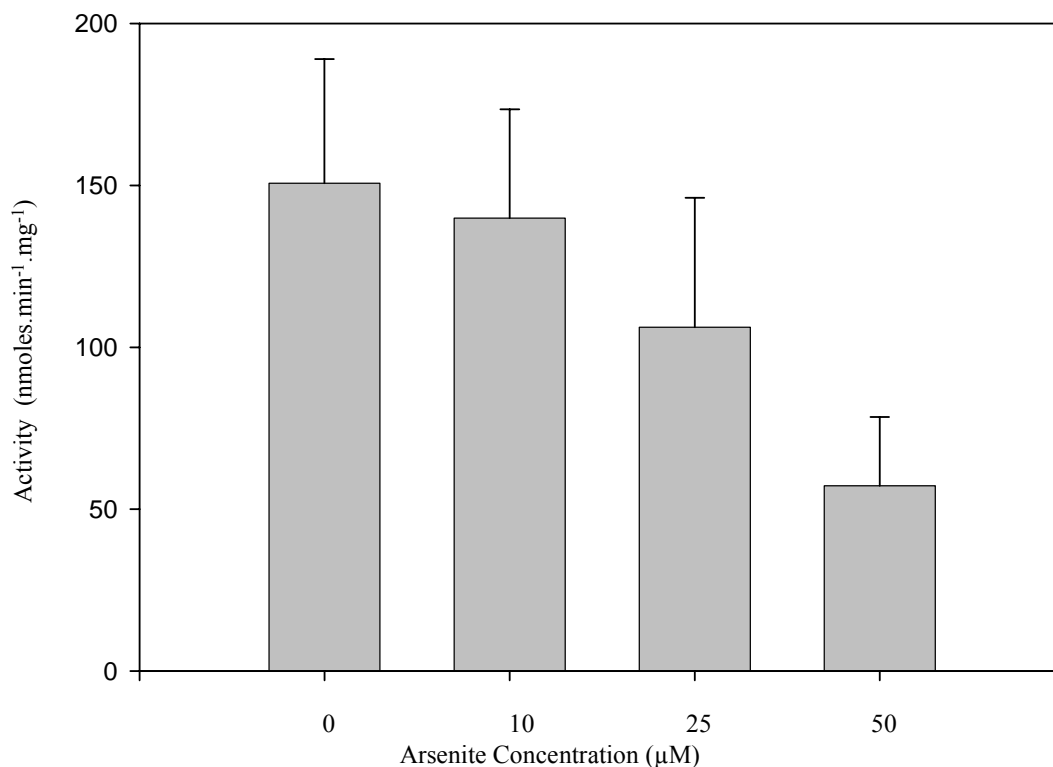


Figure 9: Plot of Activity (nmol.min⁻¹.mg⁻¹) versus $(GS)_2AsOH$ concentration. SPS was anaerobically incubated with $(GS)_2AsOH$ and the activity was analyzed on a spectrophotometer. The change in absorbance at 365 nm was recorded. Clearly, $(GS)_2AsOH$, at the concentrations used, acts as an inhibitor of SPS activity. Each point represents the average of three independent experiments.

DNA sequence analysis of SelD gene showed that human SPS 2 contains a SeCys residue at the N terminal region proximal to a Cys residue (Figure 3. Conserved N-terminal domain sequence). Since SPS 2 contains a UXC motif, it would be best to test the assays using a human SPS or the closest form to the human SPS. However, the expression of human SPS1 and SPS2 in *E. coli* has been problematic (Tamura, T., unpublished data). Therefore, we attempted to express and purify a model enzyme, SPS from *Haemophilus influenzae* which contains a SeCys.

3.2- Attempt to purify active HI SPS

Haemophilus influenzae SPS which contains a SeCys (Figure 3) has been expressed and purified in a previous study (Lacourciere, G.M., Stadtman T.C., 1999). Because HISPS resembles the human SPS2, we decided to express and purify this as a model for human SPS2. HISPS was expressed in *E. coli* and purified in three steps in the following order: Phenyl Sepharose chromatography, DEAE Fast Flow anion-exchange chromatography and finally gel filtration chromatography. During each step of the purification, we used SDS-PAGE analysis and western blots to determine the fractions to be pooled. Figure 10 shows fractions from the gel filtration that were pooled to test for SPS activity using the coupled assay.

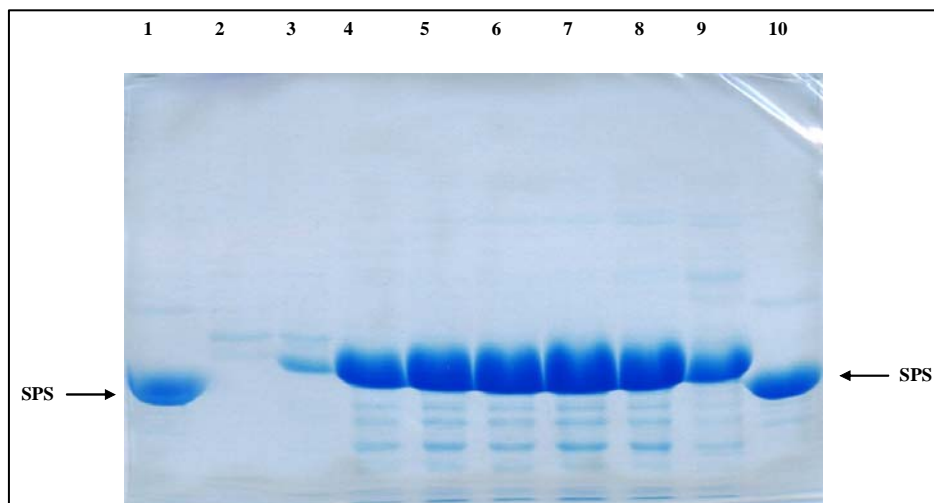


Figure 10: SDS-PAGE analysis of fractions collected after gel filtration chromatography. On lanes 1, 10, purified *E.coli* SPS was used as a marker. Lanes 3-9 show the degree of purity of HISPS in each fraction. We obtained 110 mg of about 98 % pure HISPS.

Activity of the purified HI SPS was tested using the same coupled assay that was used to follow NaHSe-dependent activity of *E.coli* SPS. We did not detect any activity with the purified HISPS. To determine whether or not a SeCys residue is present in this purified HISPS, we prepared the protein (HISPS) for X-ray absorption analysis. XAS analysis carried out in the laboratory of Dr. Robert Scott (University of Georgia) showed that HISPS preparation did not contain appreciable quantities of Se. We are unsure why this preparation does not contain selenium and has no activity.

We demonstrated that arsenite inhibits SPS and that the inhibition is competitive to selenide, the substrate. To investigate our hypothesis which states that a possible mechanism of arsenic toxicity might be the inhibition of selenium bioavailability, we decided to follow selenium incorporation into selenoproteins.

3.3- Selenium 75 (^{75}Se) labeling

It has been shown that trivalent arsenicals induce the production of ROS in Chinese Ovary Cells (Wang, T.S. et al., 1996), acute promyelocytic leukemia cells (Jing, Y. et al., 1999), and human keratinocyte cells (HaCaT) (Shi, H., et al., 2004). Based on our hypothesis, this increase is due to a decrease of the level of active selenoenzymes in the cell. HaCaT cell line is a spontaneously transformed human epithelial cell line from adult skin with unlimited growth potential; it is similar to normal skin cells.

To determine if arsenite has an effect on Se incorporation, we labeled an arsenite-treated culture of HaCat cells with the radioactive form of selenium (sodium selenite), ^{75}Se . To trace Se incorporation into selenoprotein, the cells were labeled with 10 μCi ^{75}Se radioisotope for 8 hours. Cells were then harvested, washed and lysed by sonication as described in the “Materials and Methods” section. Equal concentrations of cytosolic protein were analyzed by SDS-PAGE (Figure 11). Figure 12 shows a decrease in Se incorporation in a concentration-dependent manner. Arsenite treatment of HaCaT cells prevents incorporation of Se into selenoproteins in a concentration-dependent manner. The higher the arsenite concentration, the lower the radioactivity detected, hence, arsenite affects Se incorporation into the cell.

Based on this finding, we conclude that trivalent arsenicals affect the bioavailability of Se thus leading to a decrease in selenoproteins at a cellular level. To be sure the observed effects are specific to selenoproteins, total protein biosynthesis was analyzed.

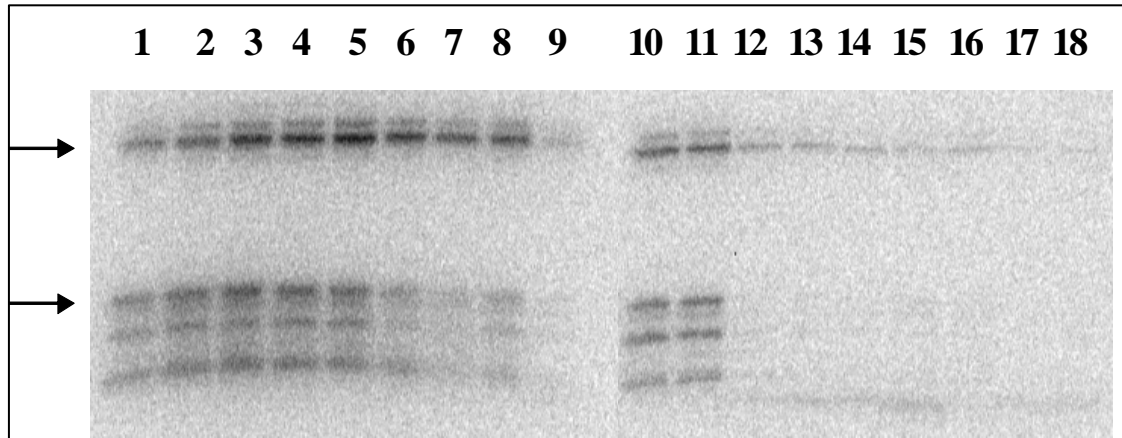


Figure 11: SDS-PAGE of ^{75}Se -Selenite radiolabeled keratinocytes treated with sodium arsenite. The keratinocytes were treated with increasing concentrations of arsenite from 200nM to 6 μM for 8 hours. Protein extracts were resolved on 12% SDS-PAGE and the labeled proteins were analyzed using a phosphorimager. Lanes 1, 2, 10, 11: controls. Lanes 3, 4: 200 nM Arsenite. Lanes 5, 6: 500 nM Arsenite. Lanes 7, 8: 1 μM Arsenite. Lanes 9, 12: 2 μM Arsenite. Lanes 13, 14: 4 μM Arsenite. Lanes 15, 16: 6 μM Arsenite. Lanes 17, 18: 8 μM Arsenite

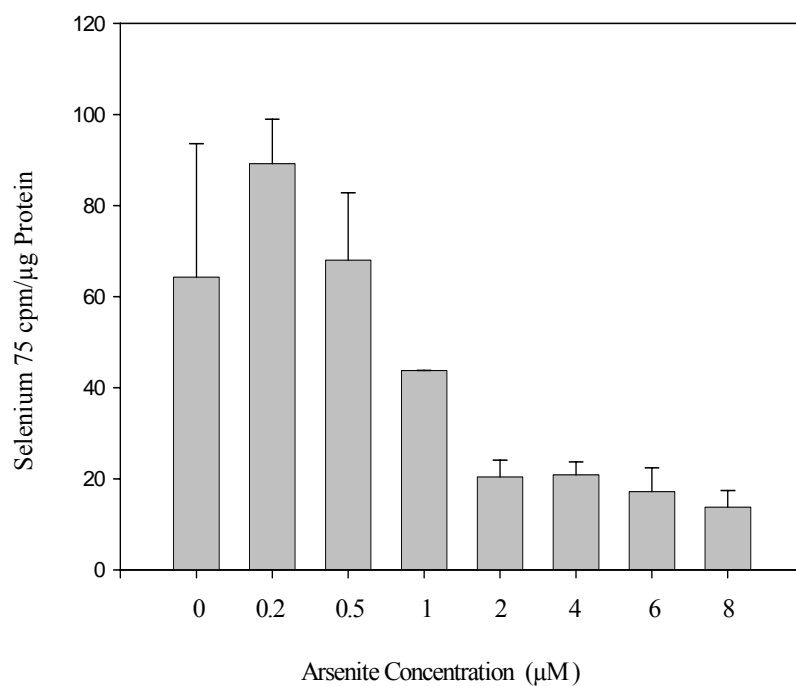


Figure 12: Arsenite treatment prevents selenium incorporation into selenoproteins. The level of radioisotope Se per microgram of cell protein was determined using a Perkin Elmer gamma counter.

3.4- Arsenite treatment of keratinocytes does not affect total protein synthesis

Arsenite treatment of HaCaT cell culture lead to a decrease in the incorporation of Se into selenoproteins. To determine whether the effect of arsenite on HaCat cells is specific to Se incorporation or extended to total protein synthesis, we treated the cells with different concentrations of arsenite and radiolabeled them with Sulfur 35 mixture of methionine and cysteine (^{35}S) for 8 hours to follow new protein synthesis. Cells were harvested and lysed by sonication. As indicated by the figure 13 and the graph on figure 14, arsenite has no effect on total protein synthesis. This confirms that the effect of arsenite is specific to incorporation of Se into selenoproteins. Although good labeling of cellular proteins was demonstrated using ^{35}S -Met-Cys mix, we also determine whether arsenite was affecting proliferation of the cells under these conditions.

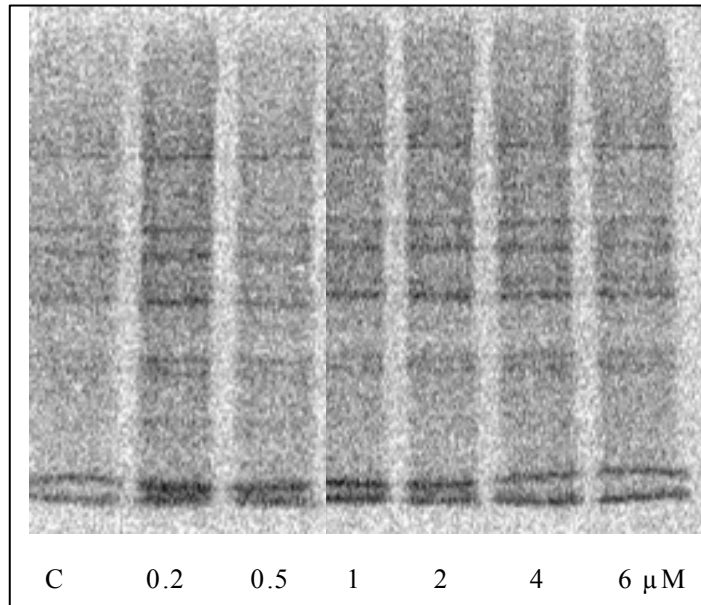


Figure 13: SDS-PAGE after ^{35}S labeling of HaCaT keratinocytes. The keratinocytes were treated with increasing concentrations of arsenite from 200 nM to 6 μM for 4 hours. Newly synthesized proteins were labeled during arsenite treatment by ^{35}S methionine/cysteine. Protein extracts were resolved on 12% SDS-PAGE and the gel was developed using a phosphorimager.

Lane1: control - No arsenite added. Lane 2: 200 nM Arsenite. Lane 3: 500 nM Arsenite. Lane 4: 1 μM Arsenite. Lane 5: 2 μM Arsenite. Lane 6: 4 μM Arsenite. Lane7: 6 μM Arsenite

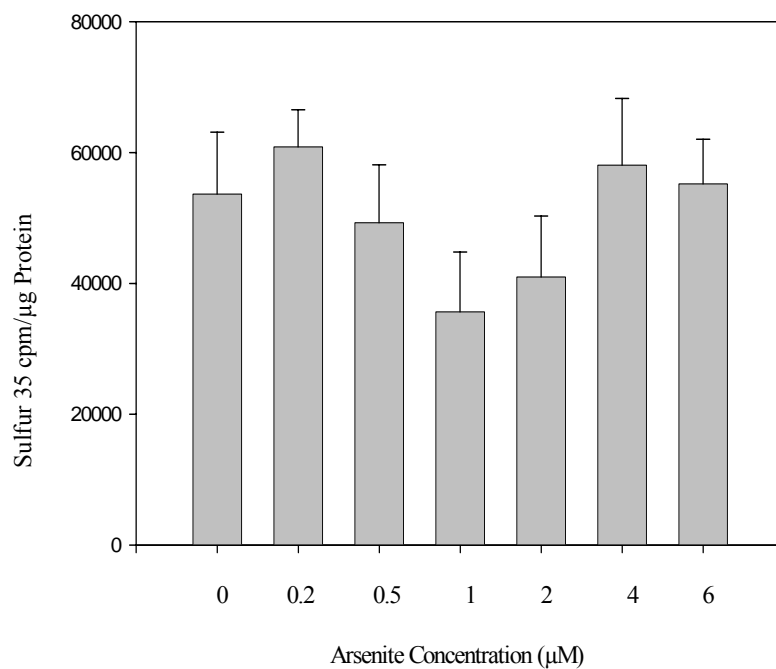


Figure 14: Measure of Sulfur incorporated into Met/Cys during new protein synthesis. HaCaT culture was labeled with ^{35}S , and then treated with increasing concentrations of sodium arsenite. The cpm/μg of protein was determined using liquid scintillation.

3.5- Arsenite does not affect keratinocyte proliferation

The previous experiments showed that treatment of HaCaT cells using micromolar concentrations of arsenite for a relatively short period of time does not affect protein synthesis.

To confirm the viability of the keratinocytes in the presence of arsenite at these concentrations (up to 16 μM), we did an MTT cell proliferation assay. The keratinocytes (4000 cells per well cultured as a monolayer) were incubated with arsenite for 24 hours prior to MTT treatment. The results presented in figure 15 show a slight decrease in proliferation with 3 and 6 μM arsenite followed by a recovery with 12 μM . At 14 μM , the cell growth becomes stable. Overall, the cell proliferation was not significantly affected by the concentrations of arsenite used.

In conclusion, these labeling experiments and cell proliferation assay confirmed that although arsenite does not affect keratinocytes proliferation in DMEM, it inhibits incorporation of radiolabeled selenium. As a control then we decided to grow the cells in defined keratinocyte medium and test the proliferation rate upon treatment with arsenite.

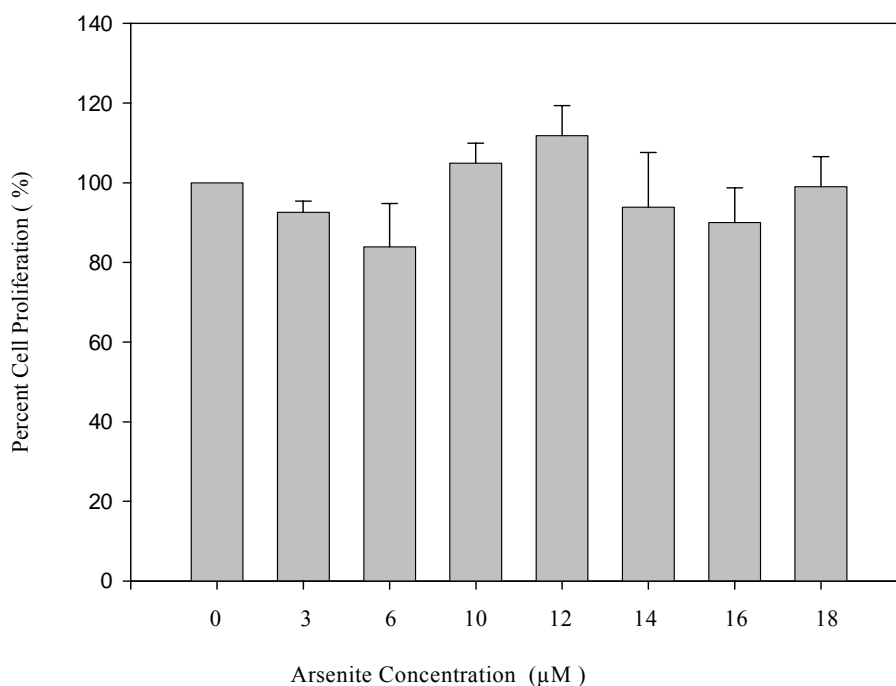


Figure 15: MTT Cell Proliferation Assay in DMEM. The keratinocytes seeded at 4000 cells per well were cultured in a 96-well plate for 24 hours and subsequently exposed to increasing concentrations of sodium arsenite for an additional 24 hours. After addition of MTT (4 hours incubation), followed by a solution of SDS-HCl for an overnight incubation, the absorbance was read at 570 nm.

In DMEM supplemented with serum, other sources of selenium such as selenoprotein P are available to the cell whereas in the defined keratinocyte medium, only 10 nM of sodium selenite is present. The composition of this defined medium will allow us to control the Se supply to the cell in order to detect any effect of higher concentrations of Se in an arsenite-treated keratinocyte culture. To determine the effect of selenite on the keratinocytes proliferation, the cells were grown in defined medium. A subculture was supplemented with 50 nM and 100 nM sodium selenite for 24 hours before exposure to the same concentrations of arsenite as reported above. Clearly, arsenite slows cell growth in defined medium as shown by figure 16A. Here the proliferation was partially recovered after addition of 50 nM selenite (Figure 16B) and completely with 100 nM (Figure 16C).

Using a defined keratinocyte medium with known concentrations of available selenite, our results showed that with certain concentrations (up to 100 nM), the effect of arsenite is reversed.

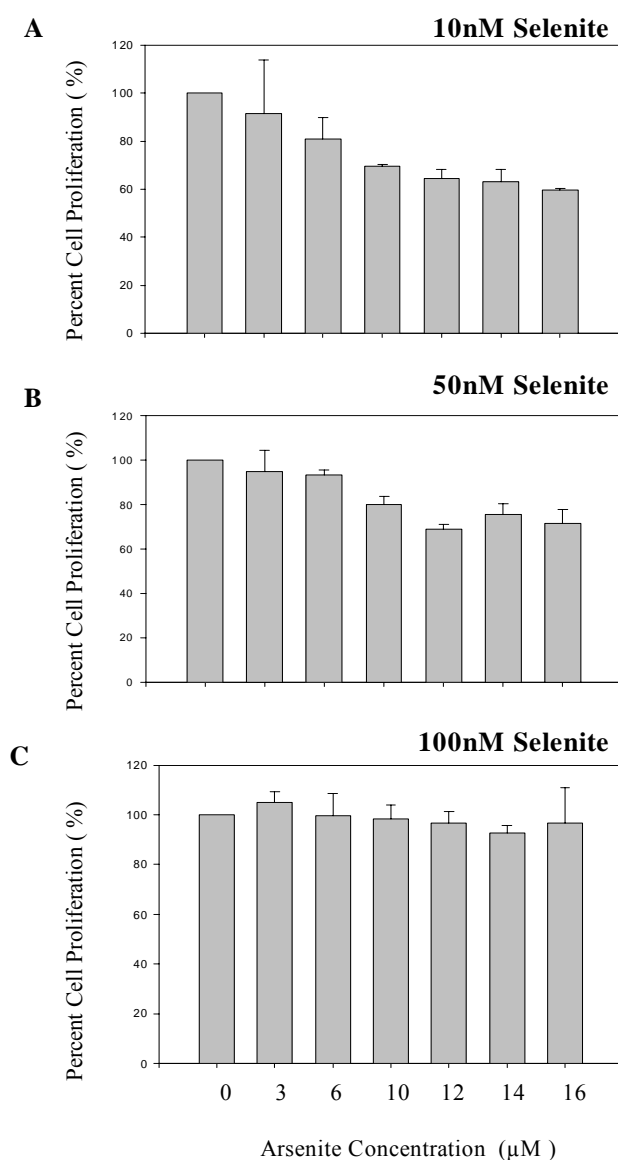


Figure 16: Effect of arsenite on cell proliferation. The keratinocytes were grown in defined medium containing 10, 50 and 100 nM sodium selenite for 24 hours and then treated with increasing concentrations of sodium arsenite for an additional 24-hour period. After addition of MTT and SDS-HCl the absorbance was read at 570 nm. The percent cell proliferation was calculated based on the untreated (no arsenite) sample. With 50 and 100 nM selenite, there is a protective effect of selenite against arsenite.

3.6- Long term arsenite treatment of HaCaT with low levels of sodium arsenite

Epidemiological studies have shown that chronic exposure to arsenic contributes to skin, lung, and bladder cancer (Smith, A.H., 1998, Schwartz, R.A., 1997, Yeh, S., 1973). There is a link between high levels of arsenite in drinking water and cancer. Also, studies demonstrated high As/Se ratio in Black-Foot Disease patients (Wang, C.T., 1993). Glutathione peroxidase functions to neutralize peroxides and oxidized membrane lipids. Thioredoxin reductase is a key enzyme in the control of cell proliferation and redox balance. Thus active Gpx and TrxR are essential to the cell's defense against reactive oxygen species. To mimic the long term exposure to trivalent arsenicals using a cell culture model, we cultured HaCaT cells in DMEM continuously for 6 weeks in the constant presence of sodium arsenite and then determined the effect of arsenite on the cells. After 6 weeks of cell culture (4 passages) without arsenite and with 0.5 and 1 μ M arsenite the cells were harvested and disrupted by sonication. The cell extract was clarified by centrifugation. The protein extract was then tested for Gpx and TrxR level by western blot and enzyme activity by spectrophotometry.

The long term arsenite treatment under our conditions did not show any morphological effects on the cells (Figure 17). The western blots show no change in the levels of Gpx and TrxR levels as presented on figure 18. Glutathione peroxidase and thioredoxin reductase, two important antioxidant selenoproteins in the cell extract were tested for activity. The activity assays confirmed the constant level of protein previously observed on figure 18. There was no significant change of the enzymes activity over time (Figures 19, 20).

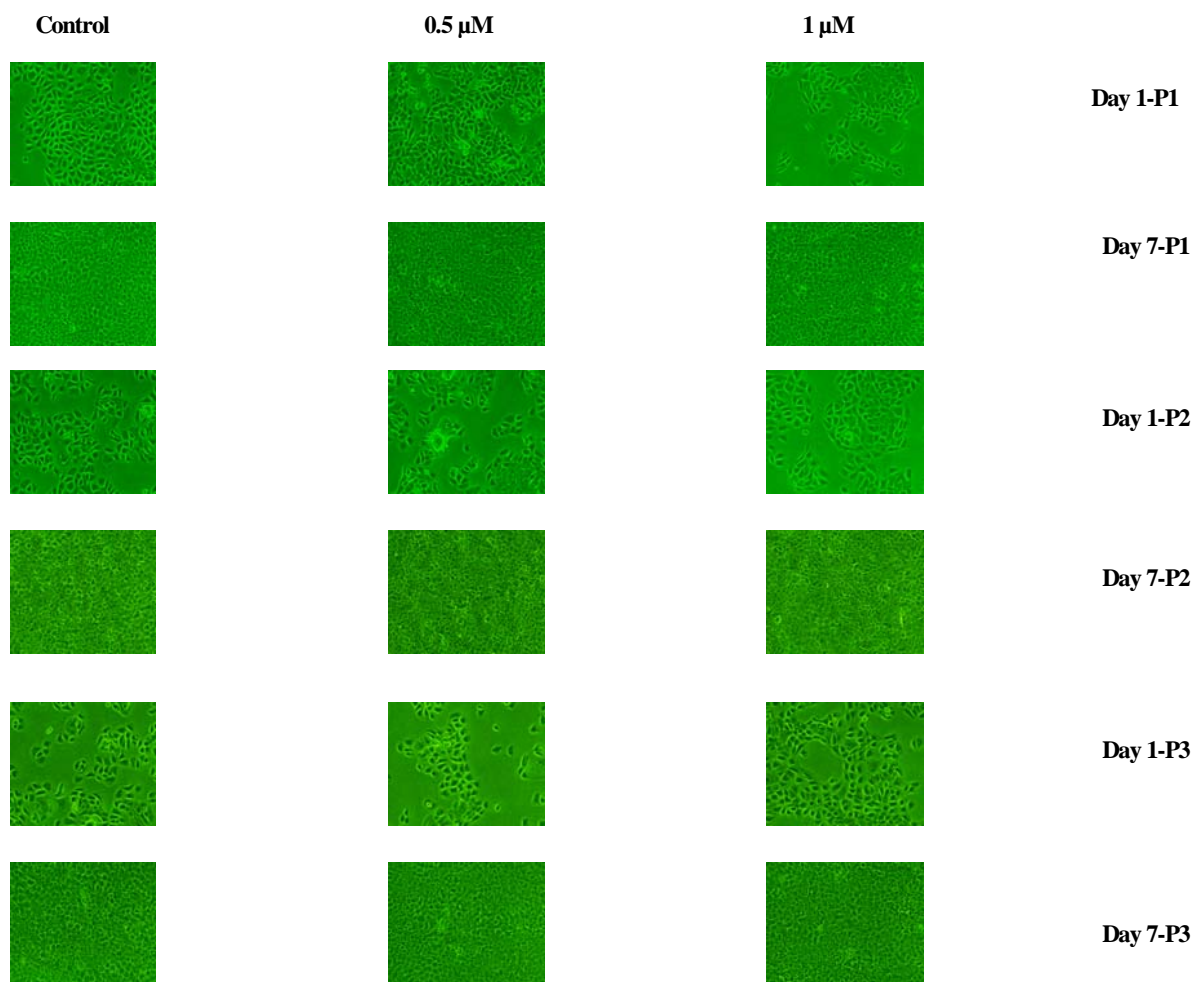


Figure 17: HaCaT treated with 0.5 and 1 μM sodium arsenite after six weeks and four passages.

These concentrations do not appear toxic to the cells.

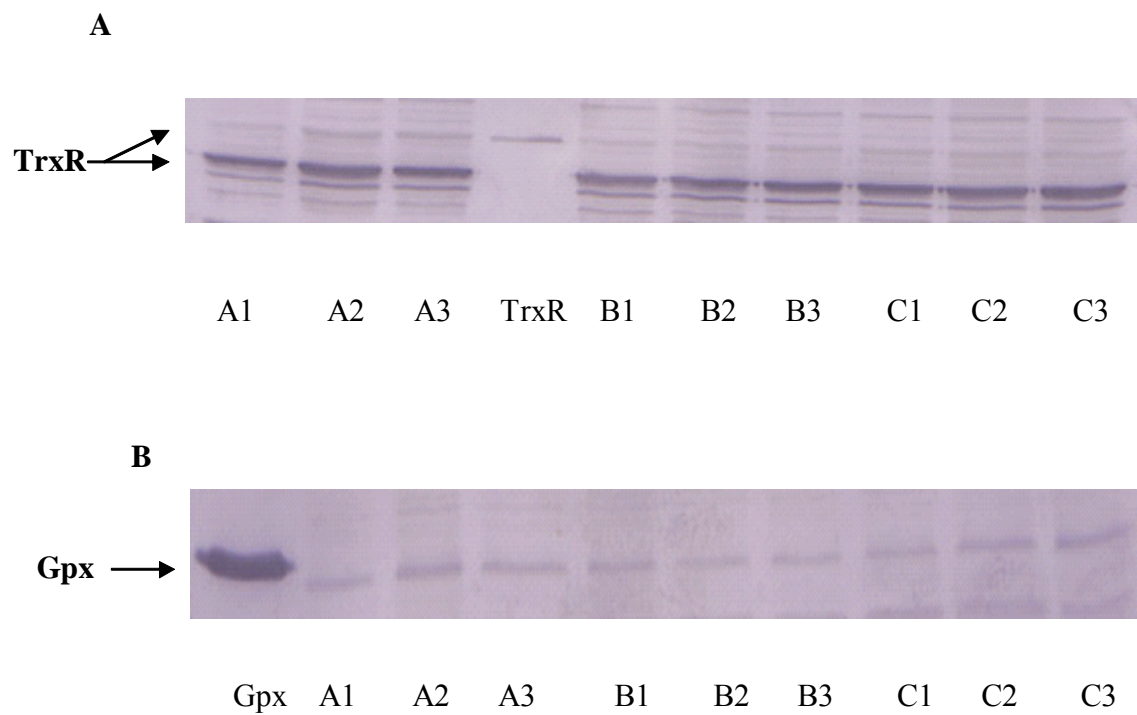


Figure 18: Immunoblot analyses of TrxR (A) and Gpx (B) levels after long term exposure (6 weeks) of keratinocytes (HaCaT) to 0.5 and 1 μ M of sodium arsenite. A1-3: Control- No arsenite. B1-3: 0.5 μ M of sodium arsenite. C1-3: 1 μ M of sodium arsenite

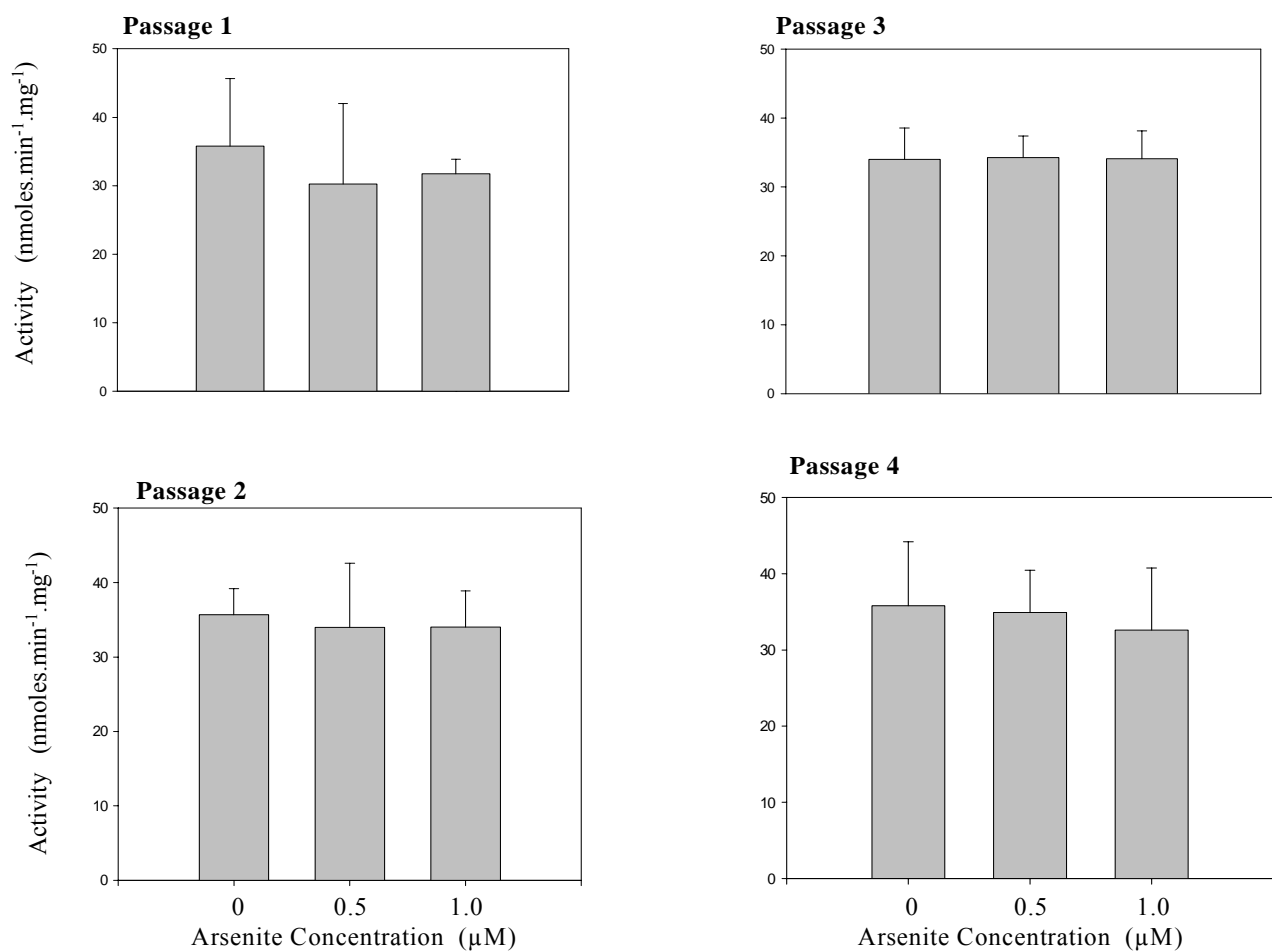


Figure 19: Gpx Activity Assay on long term arsenite-treated HaCaT culture. The cells were treated with 0.5 and 1 μM of sodium arsenite for 6 weeks (4 Passages) and then harvested. The cell extract was used (50 μg) to test Gpx activity by spectrophotometry. The change in absorbance at 340 nm was recorded and the activity calculated. A long term arsenite treatment does not affect Gpx activity.

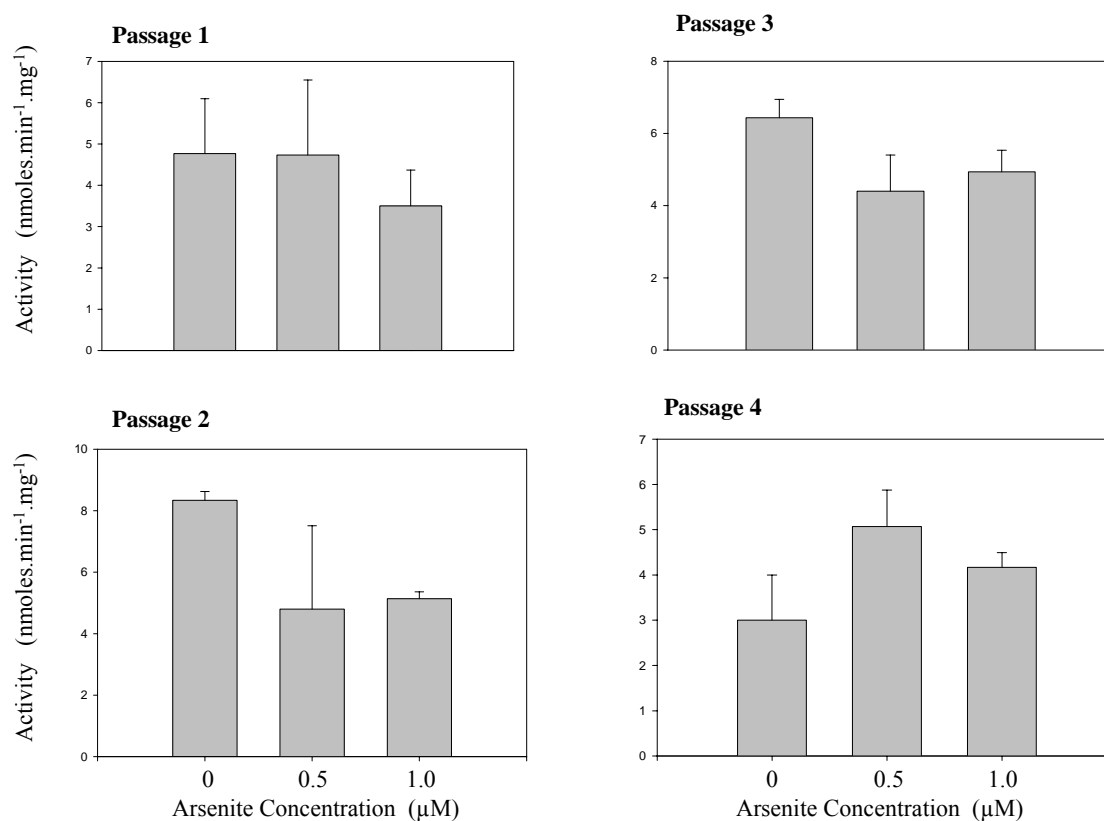


Figure 20: TrxR Activity Assay on long term arsenite-treated HaCaT culture. The keratinocytes were treated with 0.5 and 1 μM of sodium arsenite for 6 weeks (4 Passages) and then harvested. The cell extract was used (200 μg) to test trxR activity by spectrophotometry. The change in absorbance at 340 nm was recorded and the activity calculated. TrxR activity decreased with 1 μM the first 2 weeks of culture, then with 0.5 μM the following weeks to end up increasing at the last week of culture.

In summary, our results showed that arsenite induced no morphological changes of the keratinocytes after continuous culture in arsenite-containing medium. Also, arsenite did not affect Gpx and TrxR at the protein level as well as the activity level.

In DMEM supplemented with fetal bovine serum, several selenium sources are available to the cell. The keratinocytes might be using an alternate pathway that consumes a different selenium source to produce normal level of selenoproteins. This led us to investigate whether or not higher concentrations of arsenite for a shorter period of time will affect HaCaT cells and the selenoproteins level.

3.7- Short term arsenite treatment in DMEM

To determine if higher concentrations have a more significant effect on selenoproteins, we cultured and treated the keratinocytes with 2 μ M arsenite in DMEM for 96 hours. At each 24-time point, we harvested untreated and treated cells. After 4 days, we analyzed Gpx and TrxR levels by western blot. Figure 21 demonstrates that short term arsenite treatment of keratinocytes has still no effect on Gpx and TrxR levels.

Glutathione peroxidase and thioredoxin reductase activity assay also did not show any change compared to the untreated control cells (Figure 22). Although there is a downward trend of Gpx activity at day 2, the decrease is not significant. Overall short term arsenite treatment does not affect Gpx activity. Hence, we decided to control and limit the Se source by using a defined keratinocyte medium.

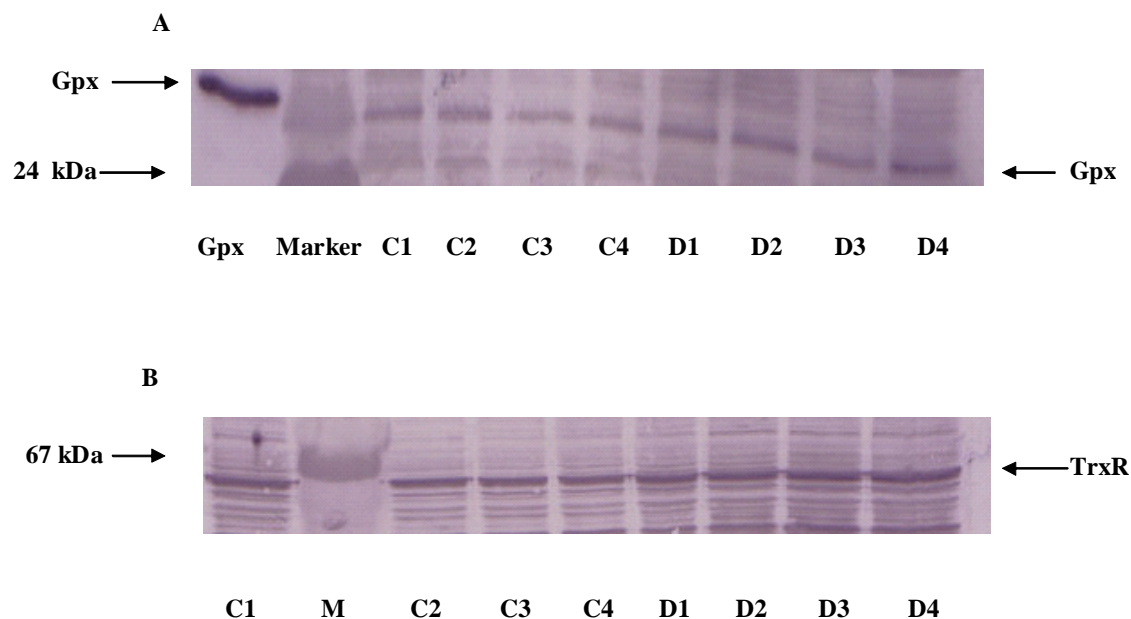


Figure 21: Analyses of Gpx (A) and TrxR (B) levels after short term exposure (4 days) of keratinocytes (HaCaT) to 2 μ M of sodium arsenite. Protein extracts were resolved on 12% SDS-PAGE and analyzed by immunoblot using Gpx and TrxR antibodies. Arsenite treatment had no effect on Gpx and TrxR levels under the studied conditions. C1-4: Control- No arsenite. D1-4: 2 μ M sodium arsenite. D1: After 24 hours. D2: After 48 hours. D3: After 72 hours. D4: After 96 hours.

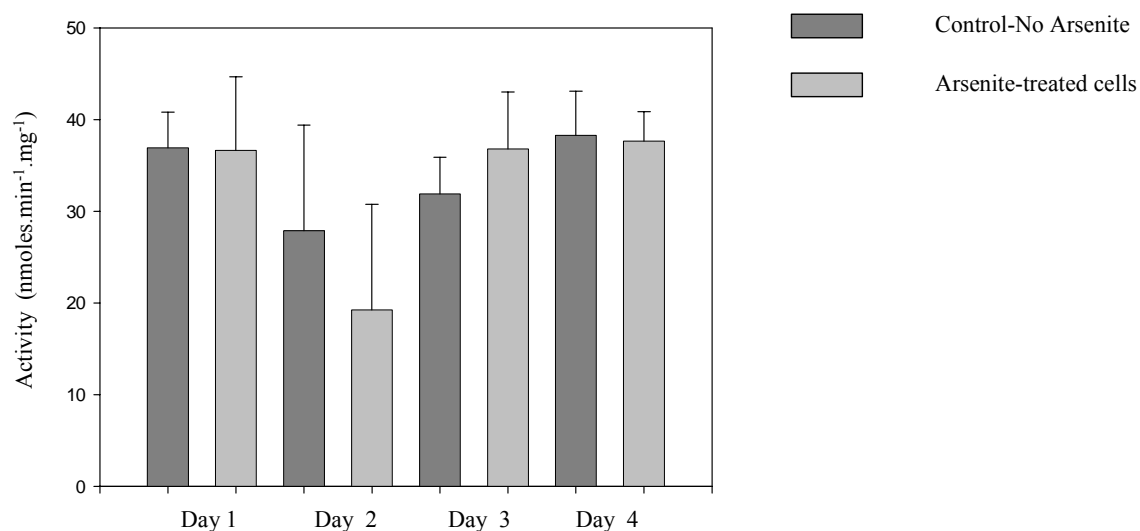


Figure 22: Gpx Activity Assay on short term arsenite-treated HaCaT culture. The keratinocytes were grown in DMEM-10 % FBS then treated with 2 μ M of sodium arsenite for four days and then harvested. The cell extract was used (50 μ g) to test Gpx activity by spectrophotometry. The change in absorbance at 340 nm was recorded and the activity calculated.

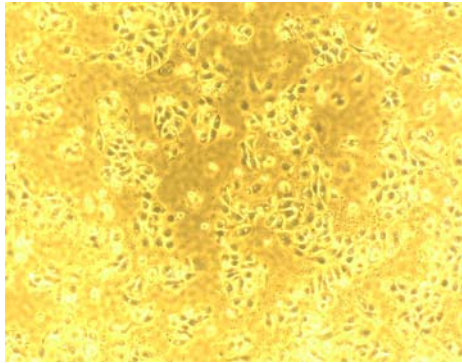
3.8- Short term arsenite treatment in defined keratinocyte medium

The keratinocytes have, in DMEM, all the nutrients necessary to grow. In consequence, the cells might use an alternate selenium supply present in serum. The Se depletion due to arsenite treatment seen in radioisotope labeling may be compensated by selenoprotein P in the serum, and this may bypass the effect of arsenicals.

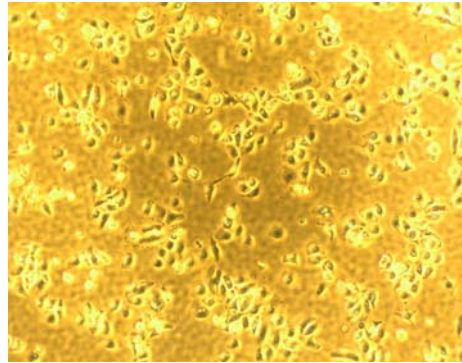
To rule out the involvement of selenoprotein P in the FBS as a selenium source, a basal serum-free medium with trace of sodium selenite (10 nM) was used to conduct another short term experiment with 2 μ M of arsenite or 4 μ M for 72 hours.

Our results presented in figure 23 showed that no change occurred in the cell morphology or cell density during the three-day treatment with 2 μ M of arsenite. The western blot analysis (Figure 24) confirmed the results by showing steady Gpx and TrxR levels. This led us to increase the concentration of arsenite to 4 μ M in the culture.

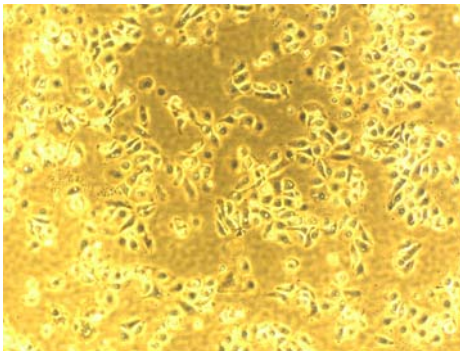
Control



Day 1



Day 2



Day 3

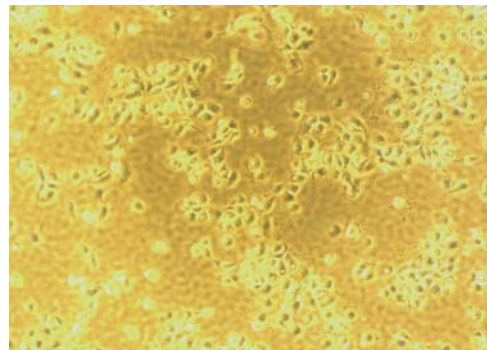


Figure 23: Keratinocytes culture in defined medium. The cells were treated with 2 μ M sodium arsenite for 3 days with no change in cell morphology and cell density.

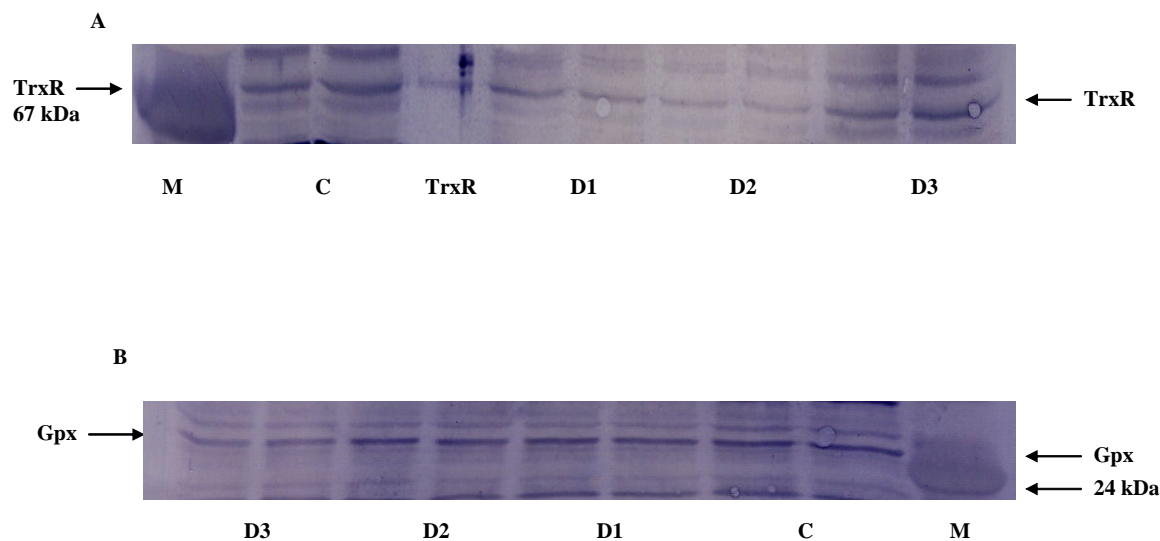


Figure 24: Analyses of Gpx (A) and TrxR (B) levels after short term arsenite treatment. The keratinocytes (HaCaT) were cultured in defined medium, and then exposed to sodium arsenite for 3 days. Protein extracts were resolved on 12% SDS-PAGE and analyzed by immunoblot using Gpx and TrxR antibodies. C1-2: Control- No arsenite. D1-3: 2 μ M sodium arsenite. D1: After 24 hours. D2: After 48 hours. D3: After 72 hours.

3.9- Short term arsenite treatment in defined medium- 4 μ M arsenite

Four micromolar arsenite would not be considered a physiologically relevant concentration but we decided to use such amount in order to trigger enough cell response to study the effect of arsenite on HaCaT.

Our results showed no change in cell culture (Figure 25) which was confirmed by the western blot analysis of two proteins marker of oxidative stress, Gpx and TrxR. Their level stayed steady through the whole experiment (72 hours) (Figure 26). Also, 4 μ M of sodium arsenite did not generate any significant change of Gpx activity over 72 hours (Figure 27).

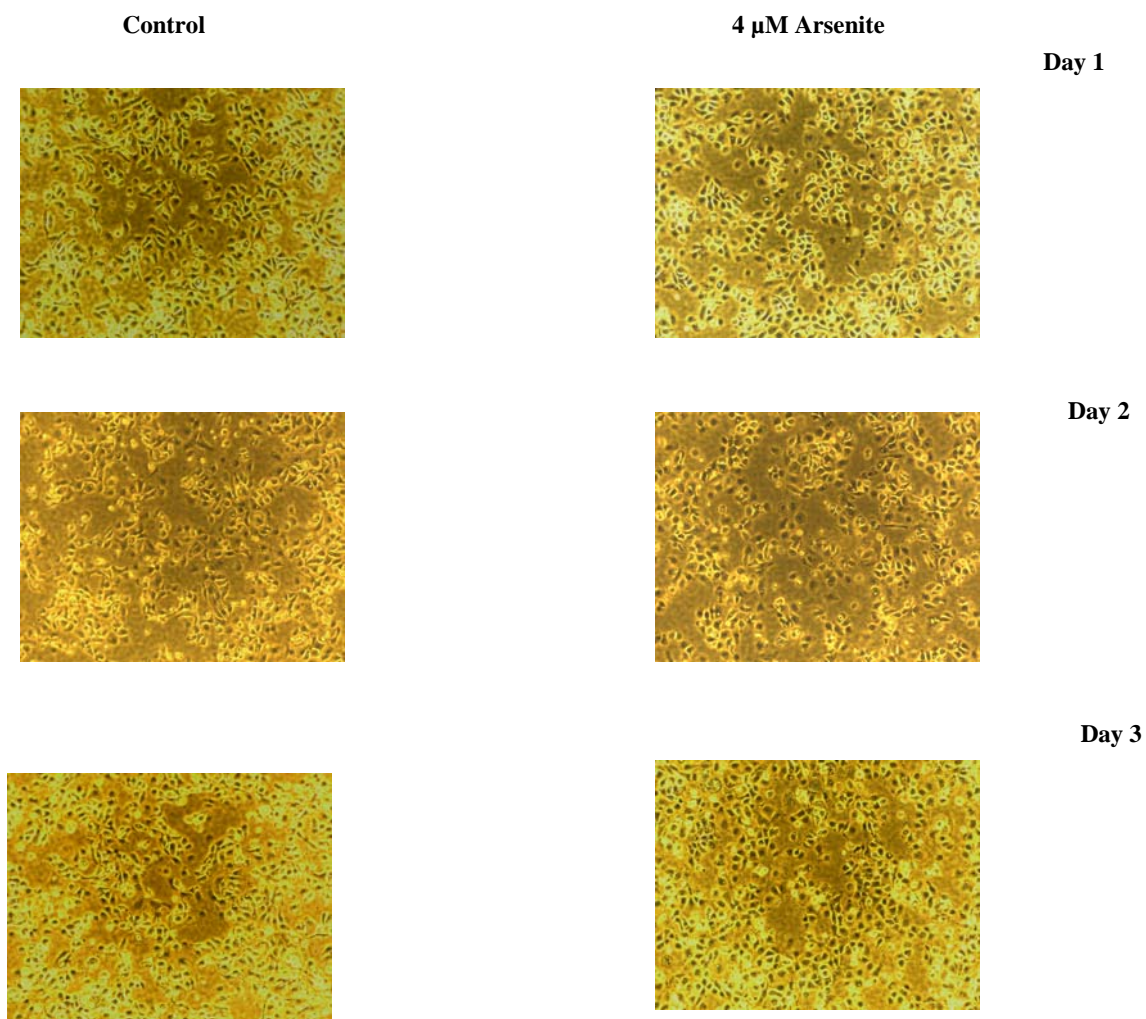


Figure 25: Keratinocytes culture in defined medium. The cells were treated with 4 μ M sodium arsenite for 3 days with no change in cell morphology and cell density.

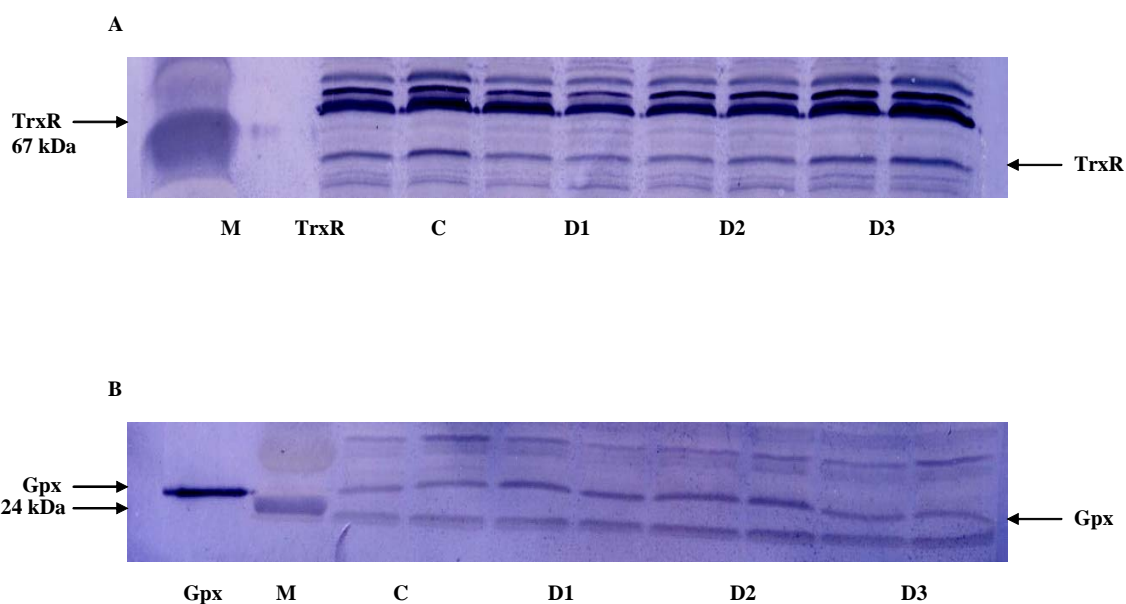


Figure 26: Western Blot analyses of Gpx (A) and TrxR (B) levels after short term arsenite treatment (4 μM). The keratinocytes (HaCaT) were cultured in defined medium, and then exposed to sodium arsenite for 3 days. C: Control- No arsenite. D1-3: 4μM sodium arsenite. D1: After 24 hours. D2: After 48 hours. D3: After 72 hours. M: Protein marker

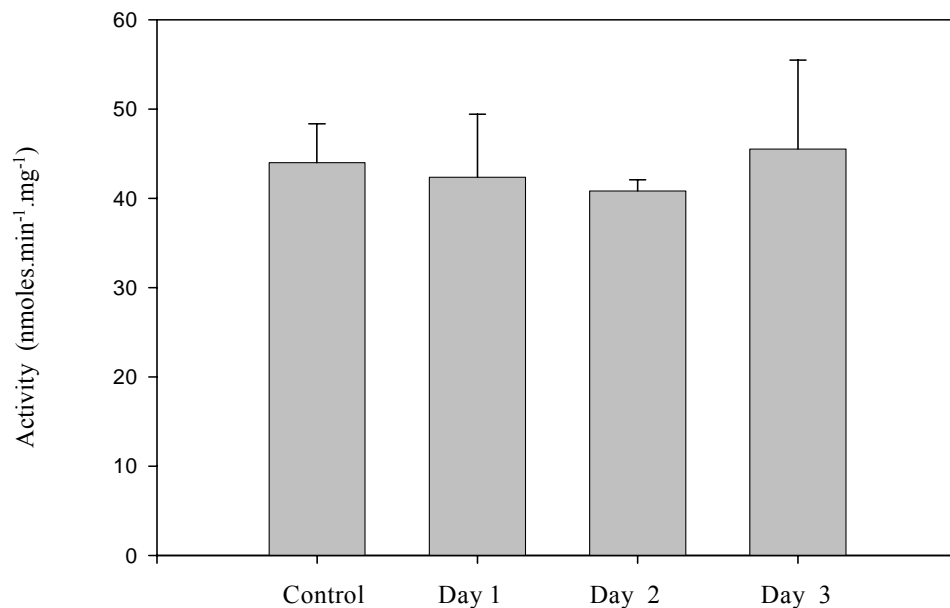


Figure 27: Gpx Activity Assay on short term arsenite-treated HaCaT culture. The keratinocytes were grown in defined medium then treated with 4 μ M of sodium arsenite for four days and then harvested. Fifty μ g of the cell extract was used to test Gpx activity. The change in absorbance at 340 nm was recorded and the activity calculated as indicated in the “Materials and Methods” section.

CHAPTER FOUR: DISCUSSION

4.1-Spectrophotometric assay for SPS

Inorganic selenium is inserted into proteins as the rare 21st amino acid selenocysteine that is essential for the catalytic function of selenoenzymes. It has been shown that selenium is required in several mammalian enzymes with roles in defense against reactive oxidative species (Carlson, B.A., et al., 2004). Selenoproteins such as Gpx, TrxR and SPS with known enzymatic functions contain selenocysteine in their catalytic center (Tamura, T. , Stadtman, T. C., 1996, (Arner, E. S., Holmgren, A., 2000, Lee, S. R. et al., 2000, Flohe, L. et al, 1973, Brigelius-Flohe, R., 1999, Chambers, I. et al. 1986, Kryukov, G. V. et al. 2002). Selenoproteins incorporate SeCys cotranslationally into the polypeptide chain by a complex mechanism (Low, S.C. et al., 1996) that is only recently being better understood.

SPS is required for the synthesis of selenoproteins in both prokaryotes and eukaryotes. SPS uses a reduced form of selenium (NaHSe in the in vitro assay) and ATP to generate selenophosphate. Previous assays for SPS (Veres, Z., et al, 1992) were hindered by the use of [¹⁴C] ATP and thin layer chromatography (TLC). The SPS assay is a sensitive coupled enzymatic assay described by Webb (Webb, M.R., 1992) for detecting inorganic phosphate and to monitor the kinetics of phosphate release by selenophosphate synthetase in solution through formation of a chromophoric product. We have incorporated this coupled assay to study the kinetics of SPS. The reagent used in this assay are easily oxidized by air, hence the importance of working under strictly anaerobic conditions. Veres's group findings corroborated the above assertion. In their study of SPS activity, they were able to lower the K_m for NaHSe from 46 μ M

to 7.3 μM by maintaining strictly anaerobic conditions (Veres, Z., et al, 1994). [^{14}C] ATP TLC assay measured the selenide-dependent formation of AMP from ATP after separation of ^{14}C labeled nucleotides by TLC and required prolonged incubation time and higher levels of enzyme, hence the importance of developing an improved and sensitive spectrophotometric assay. The improvement of the method is based on the ability to measure initial rates (V_0) more easily.

Using this coupled assay in our study, we have determined the apparent K_m value for NaHSe as 7.8 μM . This is similar to previously reported value using a different method. Our spectrophotometric assay has proven to be more useful for initial rate analysis and may lead to a better understanding of other SPS enzymes such as human SPS 1 and 2 in future studies.

4.2-Arsenite inhibits SPS

Arsenic has been implicated as a contributing factor in carcinogenesis (Hughes, M. F., 2002). Biologically, the trivalent sodium arsenite is more carcinogenic than the pentavalent form sodium arsenate (Barrett, J.C. et al., 1989). Cultured mammalian cells have provided some possible mechanisms by which arsenic and arsenical compounds may exert a carcinogenic activity. Based on known interactions of As with vicinal dithiols and the fact that selenophosphate synthetase also contains a proximal cysteine motif (Figure 3), we have tested the possibility that arsenite and/or $(\text{GS})_2\text{AsOH}$ (AsIII) would inhibit SPS.

Arsenite, a trivalent arsenical known to bind to vicinal and proximal dithiols was found to inhibit *E. coli* SPS. Amino acid substitutions in a conserved N-terminal region by Kim et al. using site-directed mutagenesis have proven that Cys-17 is vital to the enzyme activity. They

reported a complete loss of SPS catalytic activity when Cys 17 and 19 were substituted to Ser residues (Kim, I.Y. et al., 1993). Although the structure of SPS has not been elucidated yet, based on sequence homology, Cys 17 and 19 are likely to be proximal in 3-dimensional space.

Our study has shown that sodium arsenite clearly inhibits SPS activity in a concentration-dependent manner (Figure 7). Kinetic analysis revealed a competitive inhibition with NaHSe with an apparent K_i of 3 μ M likely involving Cys 17 and 19. Since the proximal cysteine motif has been previously postulated to be involved in selenium binding during catalysis, it seems likely that arsenite is also binding to this motif. The present results give support to previous ones suggesting Se binding to the proximal cysteine motif in the conserved N-terminal region. In the light of our results, it is possible that a decrease in selenoprotein synthesis due to a decrease in SPS activity might be a molecular mechanism for arsenic-induced carcinogenesis. This hypothesis led us to the next series of experiments in a cell culture model system.

4.3-Se incorporation into selenoprotein inhibited by arsenite

To determine whether arsenite has an effect on Se incorporation *in vivo*, we radiolabeled an arsenite-treated culture of HaCat cells with ^{75}Se . Our results showed a decrease in Se incorporation (Figure 11) in a concentration-dependent manner after exposure to arsenite. The higher the concentration of arsenite, the lower the Se incorporated into the cells suggesting that arsenite may be decreasing selenium bioavailability. New selenoproteins synthesis is affected by arsenite. Such observation has been reported for the first time might be due to the inhibitory effect of trivalent arsenical on SPS. Another possibility is the formation of As-Se bound compound *in*

vivo. In fact, previous studies in rabbit have shown that co-injection of As and Se results in the formation of $[(GS)_2AsSe]^-$ which is excreted from the bile (Gailer, J. et al., 2002, Gailer, J., 2002).

Since Se incorporation was affected by arsenite, we decided to determine whether this effect is specific to selenoprotein synthesis or not. We labeled the keratinocytes with ^{35}S radioisotope in order to follow total protein synthesis. We were able to show that, under our experimental conditions, sodium arsenite effect is limited to Se incorporation and does not extend to total protein synthesis. This is consistent with our *in vitro* studies where arsenite directly inhibits SPS.

4.4-Keratinocytes exposure to arsenite

SPS is inhibited *in vitro* by arsenite and there is a decrease in new selenoprotein synthesis. To investigate the effects of arsenite for a short period of time, we cultured HaCaT cells for 3 days. Western blots analysis showed a steady level of Gpx and TrxR before and after exposure to 2 μM arsenite. When we analyzed the activity of Gpx and TrxR, we still did not see any effect. The enzymes activity was constantly normal. This led us to extend the period of exposure to arsenite.

Based on the lack of effect with the short term exposure, we cultured the HaCaT cells in DMEM for 6weeks with 0.5 and 1 μM arsenite, 4 passages. Higher concentration (4 μM) killed the cells after 2 weeks. In this study, although the cells survived, we were not able to detect any change in Gpx and TrxR levels or activities. Also, concentrations up to 16 μM of arsenite did not

affect the cell proliferation rate for short periods of time (less than 48 hours). The keratinocytes are resistant to even long term exposure to arsenite which is in contradiction with our labeling experiments and *in vitro* study.

From these results, it is possible that arsenite inhibition is overcome by the cells using an alternate selenium source present in the medium. Hence, we decided to culture the keratinocytes in a defined basal medium.

4.5-Keratinocytes culture in defined medium

In DMEM supplemented with serum, other sources of selenium are available to the cells. To control the Se supply, we cultured the HaCaT cells in defined medium which contains only one form of Se and at low level. The cells are then treated with 2 and 4 μ M arsenite for 3 days. Arsenite affected the cell proliferation rate in defined medium but failed to generate any change at the protein level as well as enzyme activity level for Gpx and TrxR.

These initial studies in defined medium have yet to show an effect on TrxR and Gpx levels, however these cells were not transitioned into defined medium, so further study is needed under these conditions.

CHAPTER FIVE: CONCLUSION

In conclusion, in this study we have demonstrated that, *in vitro*, arsenite inhibits selenophosphate synthetase by competition with the substrate, sodium hydrogen selenide. We also observed a decrease in Se incorporation proportional to the increase in arsenite concentration; yet, we couldn't detect any correlation between Se incorporation and levels and activity of two selenoproteins essential in defense against oxidative damage (Gpx, TrxR). It has been proven that oxidative damage occurs after exposure to arsenicals. Our results might suggest the existence of a secondary pathway that would counteract the inhibitory effects of arsenite by incorporating into the cell a different form of selenium whenever the primary form of choice is depleted. In consequence, our revised hypothesis is that arsenic only affects certain selenium compounds.

Studies to elucidate a possible secondary pathway of selenoprotein biosynthesis are ongoing and will allow to better understand the molecular mechanism of arsenic-induced carcinogenesis in the future.

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